Isolation and Characterization of Human IL-22-producing T Cells

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Marcin Wawrzyniak

aus Polen

Promotionskomitee

Prof. Dr. Akdis Cezmi, MD (Vorsitz)

PD. Dr. Akdis Mübeccel, MD, PhD (Leitung der Dissertation)

Prof. Dr Wenger Roland H., PhD

PD. Dr. O'Mahony Liam, PhD

Zürich, 2016

Publication list

1) Increased microRNA-323-3p in IL-22/IL-17-producing T cells and asthma: a role in the regulation of TGF-β pathway and IL-22 production.
   In revision in Allergy

2) Transcriptomic characterization of human IL-22-producing T cells by next generation RNA sequencing.
   Manuscript prepared to submit to Journal of Allergy and Clinical Immunology

3) A novel, dual cytokine-secretion assay for the purification of human Th22 cells that do not co-produce IL-17A.
   Allergy 2015; DOI: 10.1111/all.12768

Table of contents

1 Acknowledgments

On the cover of dissertation only my name appears, but a lot of people have contributed to make this possible. I would like to thank all the people who helped me in science and beside during my time at SIAF.

First of all I would like to thank my supervisor PD. Dr. Mübeccel Akdis for giving me the opportunity to do my PhD project in SIAF. Thank you for giving me the freedom to explore on my own and the same time for the guidance on every step of my PhD thesis. Thank you for patience, motivation and knowledge you have shared with me and for creating a great friendly atmosphere in our group. To Prof. Cezmi Akdis, the director of SIAF, thank you for your support and discussions and comments that helped me to improve my project and me as a researcher.

Beside my supervisors, I would like to thank the rest of my PhD thesis committee members, Prof. Dr. Roland H. Wenger and PD. Dr. Liam O`Mahony for advice, comments and fruitful discussion during PhD thesis committee meetings. I would like to acknowledge our academic collaborators, Dr. Ana Rebane and Janika Kärner and all the co-authors of paper describing the role of miRNA in Th22 cells.

I would like to thank all SIAF members, former and present that I have a pleasure to work with over the last few years. I am very grateful to: Paulina Wawrzyniak, Annelot Breedveld, Janika Kärner, Toomas Runnel, Ana Rebane, Hideaki Morita, Beate Rückert, Francesc Castro Giner, Avidan Neumann, Claudio Rhyner, Urs Ochsner, Oliver Wirz, Willem van de Veen, for direct involvement and help with my projects. To office-labmates, Hideaki Morita and Oliver Wirz for our stimulating discussions, sleepless nights that we were talking about science and all fun we had in last few years. I am also thankful to David Groeger for careful reading, commenting and proof-reading all manuscripts I have produced. For translating the summary of this thesis into

German I thank David Mirer. For helping me and my family with all paper work I thank Daniela Bert and Sandra Crameri, our great SIAF secretaries. I would like to express my special thanks to Beate Rückert, for her patience and our long discussions that helped me sort out the problems of my work. To Maciej Chałubiński with whom I have started my scientific adventure in Davos. My special thanks go as well to "polish society in Davos": Andrzej Eljaszewicz, Milena Sokołowska, Ania Głobińska, Wojciech Lipowski, Weronika Barcik, and Ania Zaleska for making me feel like I was at home.

Last but not the least, I would like to thank my family to whom this dissertation is dedicated to: my wife Paulina for supporting me in the lab but most importantly in every-day live – I could not accomplish this without you by my side, my daughter Natalia, for giving me strength to face difficulties and remind me every day that there is a life beyond science, my parents and parents in law, my sister Ania and Julek with family, Edyta and Adam with family, for being around and helping us whenever we needed you.

This work was supported by the Swiss National Science Foundation grants 32-132899, 32-140772, 32-159870 and 32-112306, the Christine Kühne-Center for Allergy Research and Education, Davos Switzerland (CK-CARE), Swiss-Polish contribution, institutional research grant IUT2-2.

2 Summary

T cells mediate adaptive immune responses via activation of other immune cells and regulation of tissue cells to eliminate bacterial, fungal and viral pathogens and maintain homeostasis. Based on cytokine production patterns, T helper cells were classically subdivided into Th1, Th2 and T regulatory cells. Recently, new subsets of T helper cells, including Th9, Th17 and Th22 cells have been described. One of the cytokines produced by Th17 and Th22 cells is interleukin-22. Because of preferential expression of IL-22R1 on cells of epithelial origin, IL-22 is a unique cytokine produced by immune cells, which acts only on tissue cells. Among all T helper cells subsets, IL-22-producing T cells are characterized to a lesser extent as compared to other cells. In this PhD thesis, I have studied human IL-22-producing T cells in depth and further characterized them.

The data demonstrated in these studies is novel in terms of three aspects. First of all, for the first time miRNA expression analysis in human IL-22-producing T cells has been performed and new miRNA-323-3p has been reported as a potential negative regulator of IL-22 production from T cells. Secondly, next generation sequencing of human IL-22-producing T cells sorted with in-house generated IL-22 secretion assay was performed and full profile of IL-22-producing T cells including potential transcription factors was provided. Finally, a novel double cytokine secretion assay that allows sorting of viable Th22 cells that do not co-produce IL-17A was generated.

Better understanding and characterization of human Th22 cells may provide novel therapeutic options in the treatment of diseases like psoriasis, atopic dermatitis, rheumatoid arthritis, where a pro-inflammatory role of IL-22 has been reported.

3 Zusammenfassung

T-Zellen vermitteln die adaptive Immunantwort über die Aktivierung weiterer Immunzellen und der Regulierung von Gewebezellen um bakterielle, pilzartige und virale Pathogene zu eliminieren und die Homöostase aufrechtzuerhalten. Basierend auf dem Zytokinproduktionsmuster, werden T-Helferzellen üblicherweise in Th1, Th2 und T-regulatorische Zellen unterteilt. Kürzlich wurden neue Untergruppen von T-Helferzellen wie Th9, Th17 und Th22 – Zellen definiert. Th17 und Th22-Zellen produzieren vorwiegend Interleukin-22. Aufgrund der bevorzugten Expression von IL-22R1 in Epithelzellen ist IL-22 ein Zytokin, welches speziell nur auf Gewebezellen wirkt. Unter den T-Zelluntergruppen sind IL-22-produzierende T-Zellen weniger gut charakterisiert als andere. In dieser PhD Thesis habe ich humane IL-22-produzierende Zellen untersucht und das Wissen über diese erweitert.

In dieser Studie werden Daten gezeigt, welche bezüglich dreier Aspekte neuartig sind. Zunächst, wurde erstmalig eine miRNA Expressionsanalyse in humanen IL-22-produzierenden T-Zellen durchgeführt und miRNA-323-3p wurde als ein potentieller negativ-Regulator für die Produktion von IL-22 in T-Zellen beobachtet. Zweitens wurde ein next generation sequencing von humanen IL-22-produzierenden T-Zellen mit einem selbst generierten IL-22 Sekretionsassay durchgeführt. Das gesamte Profil von IL-22-produzierenden T-Zellen, inklusive potentielle Transkriptionsfaktoren, wurde dabei ermittelt. Drittens wurde ein neuartiger doppel-Zytokin Sekretionsassay entwickelt, welcher das Sortieren von viablen Th22-Zellen ermöglicht, die kein IL-17A koproduzieren.

Das bessere Verständnis und die Charakterisierung von humanen Th22-Zellen könnte dazu beitragen, neue Therapiemöglichkeiten in der Behandlung von Krankheiten zu finden, in welchen IL-22 eine proinflammatorische Rolle spielt (wie Psoriasis, atopischer Dermatitis und rheumatische Arthritis).

4 Abbreviations

3` UTRs	3` untranslated regions
AD	atopic dermatitis
Ag	antigen
AGO	Argonaute protein
AhR	aryl hydrocarbon receptor
APC	antigen presenting cell
Bcl-2	B-cell lymphoma 2
C domain	constant domain
CD disease	Crohn`s disease
CDRs	complementarity-determining domains
CNS	central nervous system
c-SMAC	central – SMAC
CTL	cytotoxic T lymphocytes
CXCL	CXC chemokines ligand
DC	dendritic cell
dsRNA	double stranded RNA
EAE	experimental autoimmune encephalomyelitis
ER	endoplasmic reticulum
GM-CSF	granulocyte-monocyte colony stimulating factor
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus type 1
IBD	infalmmatory bowel disease
ICAM	intercellular adhesion molecule
IELs	intraepithelial leukocytes
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-22BP	IL-22 binding protein
IL-22R	IL-22 receptor
ILCs	innate lympoid cells
IL-TIF	IL-10-related-T cell-derived inducible factor
IRF	interferon regulatory transcription factor
ITAMs	immune-receptor tyrosine-based activation motifs
LFA	lymphocyte function-associated antigen
LPS	lipopolisaccharide

LRRs	leucine-rich repeats
MAPK	mitogen-activated protein kinase
MD2	myeloid differentiation factor 2
MHC	major histocompatibility compex
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin kinase
MyD88	Myeloid differentiation factor 88
ncRNA	non-coding RNA
NF-κB	nuclear factor kappa beta
NK cell	natural killer cell
NKT cell	natural killer T cell
NLRs	NOD-like receptors
OVA	ovalbumin
PAMPs	pathogen-associated molecular patterns
PBMC`s	peripheral blood mononuclear cells
PI3K	phosphoinositide 3-kinase
poly(I:C)	polyinosinic-polycytidylic acid
PPRs	pathogen recognition receptors
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
p-SMAC	peripheral – SMAC
PTs	palatine tonsils
RA	rheumathoid arthritis
Rag	recombination-activating gene
REG	regenerating islet-derived protein
RLRs	RIG-like receptors
RSV	respiratory syncytial virus
SMAC	supramolecular activation complex
SOCS1	suppressor of cytokine signalling 1
ssRNA	single stranded RNA
STAT	signal-transducing activators of transcription
T reg	T regulatory cel
Tc cell	cytotoxic T cell
TCR	T cell receptor
TGF	transforming growth factor

TMCs	Tonsil mononuclear cells
Th cell	T helper cell
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLRs	Toll-like receptors
TRAF	tumor necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor-inducing IFN-γ factor
UC	ulcerative colitis
V domain	variable domain

5 General introduction

5.1 Historical background

The immune system developed during evolution to ensure that multi-cellular organisms are protected from pathogens. To match the diversity of pathogens invading our body, the immune system requires a broad range of recognition and elimination mechanisms. The above mentioned protection is provided by sophisticated and powerful network of organs, tissues, cells, molecules and pathways. Immunology as a scientific discipline, grew out of the observation that individuals, who had recovered from infectious diseases were protected from them when encountered for the second time. The origin of immunology has dated back to the late 18th century when the English physician Edward Jenner observed that milkmaids that contracted cowpox disease were immune to severe smallpox. Based on this observation, in the procedure called vaccination, he inoculated people with fluid from the cowpox postule and by which protected them from smallpox. Later, Robert Koch proved that infectious diseases are caused by microorganisms. Those discoveries of Koch and Jenner; opened new possibilities of vaccinations against other diseases. In the years 1881-1886, Louis Pasteur developed protection strategies against cholera, rabies and anthrax. The need to search for and understand the mechanisms underlying protection against infectious diseases led to the development of immunology as a science.

Immune system protects the host from the invasion of pathogens. The main success in the field of immunology is that a lot of infectious diseases have now been eradicated, the others are fully controlled, organ transplantation is possible, and immune-related drugs used to treat life-threatening disorders. Nevertheless, a range of disorders can still result from defective immune responses, what is mainly seen in hypersensitivity reactions (including

allergies), autoimmune diseases, immune deficiencies and during the development of cancers [1-3].

5.2 Principles of innate and adaptive immunity

Four main tasks must be fulfilled by the immune system to effectively protect against a disease. First of all, the presence of infection must be detected in the process of immunological recognition. Later, infection must be contained and if possible completely eliminated with the help of the effector functions of immune cells. In parallel, the immune system has to self-regulate and keep its own response under control. Failure of the immune regulatory function may contribute to the development of inappropriate immune reactions, like those observed in many of the allergies or the autoimmune diseases. Lastly, individuals exposed at least once to infections agents should develop immunological memory, which will ensure stronger and faster protective immunity when facing subsequent exposure to the same pathogen. To be able to fulfill completely those tasks there are two interconnected types of immunity: innate and adaptive.

Mechanisms of innate immunity are encoded in the germline and developed earlier during evolution. The recognition strategies used in innate immunity are fast reacting, but not very precise and specific. This first line of defense is focused on prevention of infection and quick elimination of common threats via physical and chemical barriers, pathogen recognition receptors, phagocytes, and broad range of serum proteins.

On the other hand, adaptive immune system is specific for subtle molecular differences of each individual pathogen. The system relies on B and T lymphocytes, which via randomly generated receptors ensure antigen-specific responses. The fact that only few cells possess the specific receptor and those cells undergo clonal selection and proliferation upon Ag recognition. In addition, adaptive immune response takes more time to develop. The most

significant feature of adaptive immune system is ability to remember prior exposure and generate immunological memory to respond faster and with greater efficiency upon repeated exposure to the same pathogen.

The accurate development of adaptive response is dependent on innate immunity that is why both systems do not exist separately, they are dependent upon one another and cooperate for maximal effectiveness in protection of the host [1-3].

5.3 The innate immune system

The innate immune system consists of physical and chemical barriers as well as cellular responses against infection. The epithelial cells of skin and respiratory, gastrointestinal and urinary mucosal tissues are the anatomical barriers, which prevent pathogens enter the body. Formation of anatomical barrier is possible due to the fact that epithelial cells are connected with each other via tight junction molecules, and form a seal against the external environment. Besides providing a passive barrier function, the epithelial lining of mucosa also actively contribute to immunity by the production of antimicrobial peptides, reactive oxygen and nitrogen species, cytokines, chemokines and mucus [4, 5].

Despite the protection by epithelial cells, many pathogens developed strategies to by-pass these layers. The next line of defence is composed of phagocytes, namely macrophages, neutrophils and dendritic cells. Phagocytes as well as epithelial cells, via pathogen-recognition receptors (PRRs), directly recognize pathogen-associated molecular patterns (PAMPs) present on many microorganisms. PRRs as one of the most important invariant receptors of innate immunity detect and trigger effective defence against pathogens. They recognize microbial products that are very crucial for the microbes' survival, so ensuring microbes cannot adapt and evade recognition by the host. Some of PRRs, like C-type lectin receptors and scavenger receptors upon microbial binding and

recognition trigger phagocytosis. The others, particularly Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs) trigger transcription pathways of genes encoding antimicrobial peptides, interferons, cells recruiting and activating chemokines and cytokines, enzymes, all with important functions in protective immunity. Broad spectrum PRRs, located inside as well on the cell surface, ensures that the cell is able to recognize PAMPs from intracellular and extracellular pathogens [1-3].

The innate immune system not only recognizes conserved microbial structures but also DAMPs (damage-associated molecular patterns), the endogenous products releases from dying or damaged cells.

Until now 5 different classes of PRRs have been identified: Toll-like receptors, retinoic-acid-inducible gene-I-like receptors, nucleotide-binding domain and leucine-rich repeat containing gene family, C-type lectin receptors and cytosolic DNA receptors [6, 7].

5.3.1 Toll-like receptors

Toll-like receptors, the prototype PRRs, were characterized as a unique factor responsible for *Drosophila melenogaster* development and immunity [8, 9]. The spectrum of PAMPs recognized by TLRs is very broad, including nucleid acids, proteins, lipoproteins and lipids from microbes such as parasites, fungi, viruses and bacteria [10]. This membrane bound family of PRR sense PAMPs on the cell surface (TLR1, TLR2, TLR4, TLR6) as well as in the endosomes (TLR3, TLR7, TLR8, TLR9) and share a common structural element in the extracellular region called leucine-rich repeats (LRRs) [11]. The subdivision of TLRs based on their localization is related with the category of PAMPs they recognize. The group recognizing microbial membrane components is localized on the host cells surface and the group that recognize microbial nucleid acids is exclusively expressed in the intracellular vesicles including endoplasmic reticulum (ER), endolysosomes, lysosomes and

endosomes. So far, 10 functional TLRs were identified in humans (TLR1-TLR10) and 12 in mice (TLR-1-TLR13) with TLR10 being not functional and each with a distinct repertoire of specificity against PAMPs (described in Table 1) [12]. In Table 1, all known human TLRs and their corresponding ligands are shown.

Table 1. Toll-like receptors and their microbial ligands

TLRs	Ligand	Microbes	Localization
TLR1	Triacyl lipopeptides	Mycobacteria and Gram-negative bacteria	Cell surface
TLR2	Peptidoglycan Lipoproteins Zymnosan Phosphatidylserine GPI-linked proteins	Gram-positive bacteria Mycobacteria Yeast and other fungi Schistosomes Trypanosomes	Cell surface
TLR3	dsRNA Polyinosinic-polycytidylic acid (poly(I:C))	Viruses	Endosomal
TLR4	LPS F-protein Mannans	Gram-negative bacteria RSV virus Fungi	Cell surface / Endosomal
TLR5	Flagellin	Bacteria	Cell surface
TLR6	Diacyl lipopolypeptides Zymnosan	Mycobacteria and Gram-positive bacteria Yeast and other fungi	Cell surface
TLR7	ssRNA	Viruses	Endosomal
TLR8	ssRNA	Viruses	Endosomal
TLR9	CpG unmethylated dinucleotides Dinucleotides Herpes virus components Hemozoin	Bacterial DNA Herpes viruses Malaria parasite heme byproduct	Endosomal
TLR10	Unknown	Unknown	Endosomal

5.3.1.1 Cell surface TLRs

TLR4, the first described TLR, is responsible for binding to lipopolisaccharide (LPS), the main component of the cell wall from gram-negative bacteria [13]. The LPS-binding component is formed by TLR4 and MD2 (myeloid differentiation factor 2), The TLR4-MD2-LPS complex initiates the signal transduction. Recently, it has been show that TLR4 is not only involved in recognition of TLR4 but can recognize pneumolysin from *Streptococcus pneumoniae,* envelope proteins of the mouse mammary tumor and the fusion proteins of respiratory syncytial virus (RSV) [10]. Another member of TLRs family expressed on the surface is TLR2 that is responsible for recognition of lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, tGPI-mucin from *Trypanosoma cruzi,* hemagglutinin protein from the measles virus [10]. The unique feature of TLR2 is that it forms heterodimers, either with TLR1 to recognize triacylated lipopeptides or with TLR6 to recognize diacetylated lipoproteins [14, 15]. TLR5 recognize bacterial flagella [10]. Cells that express high levels of TLR5 are lamina propria dendritic cells from the small intestine (CD11c+CD11b+), which are involved in the promotion of Th17 and Th1 cell development and induction of IgA-producing plasma cells [16]. TLR10, because of its sequence similarity to TLR1, might form heterodimers with TLR2, but so far the role of TLR10 is not known [12].

5.3.1.2 Intracellular TLRs

Discovered in 2001, TLR3 was the first TLR that was shown to be involved in antiviral response [17]. Primarily, the polyinosinic-polycytidylic

acid (poli (I:C)), a synthetic analog of double-stranded RNA, which mimics a viral infection, was used to identify TLR3 ligands. Nowadays, it is well established that TLR3 mediates recognition not only of poli (I:C), but recognizes the genomic RNA of reoviruses, single stranded RNA (ssRNA) viruses like RSV virus, encephalomyocarditis virus, West Nile virus, which produce dsRNA during replication in host cells [10, 18]. The essential role of TLR3 in the activation of type I interferon (IFN). The mammalian types are designated IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), and IFN-ζ (zeta, also known as limitin) [19], as well NF-kB signalling pathways and preventing virus infection is clearly evident in TLR3 deficiency in humans, who are susceptible to herpes simplex virus type 1 (HSV-1) infection [20]. In the mouse models, it was shown that TLR3 deficient mice are highly susceptible to the infection with murine cytomegalovirus [21].

TLR7 was identified in the experiments in, which antiviral and antitumor drugs (imiquimod, resiquimod, loxirinine) were recognized by this TLR7. Additionally, TLR7 recognizes ssRNA of human immunodeficiency virus (HIV), influenza A virus and vesicular stomatitis virus [10, 22]. Plasmacytoid dendritic cells (pDC), which produce type I IFN`s after virus infections, express high levels of TLR7. Similarly to TLR7, TLR8 recognizes viral ssRNA as well.

The last of the intracellular TLR`s characterized so far, TLR9 recognizes unmethylated-CpG DNA (2`-deoxyribo (cydidine-phosphate-guanosine)) motifs, that are rare in mammalian cells but are present in bacteria and viruses. Direct activation of dendritic cells, macrophages and B cells is possible by synthetic CpG oligodeoxynucleotides, which serves as TLR9 ligands. It was shown recently that stimulation of B cells via TLR9 induce IL-10 production from B cells [23].

5.3.1.3 Signal transduction pathways and generated response

Toll-like receptors` cytoplasmic domain contains the so-called TIR domain (Toll/IL-1 receptor) that serves as the binding site for TIR domains of adaptors involved in the downstream signalling pathway. There are two main adaptors: MyD88 (Myeloid differentiation factor 88) and TRIF (TIR-domain-containing adaptor-inducing IFN-β factor) and two main sorting adaptors: TRAM, that recruits TRIF to endosomal TLR4 and TLR3, and TIRAP that recruits MyD88 to TLR2 heterodimers and TLR4. MyD88 adaptor molecule binds to most of TLRs except TLR3 to which the TRIF adaptor protein binds. In the MyD88-dependent signalling pathway MyD88 binds to TIRM domains of the plasma membrane TLR2/1, TLR4, TLR5 and induce NF-kB and MAPK pathways. Additionally, the activation of the NF-kB and MAPK in the MyD88 dependent pathways, TLR7, 8 and 9 also activate interferon regulatory factors (IRFs), that are fundamental in the transcription of type I interferon genes.

Alternatively, in the TRIF-dependent signalling, TLR3 and endosomal TLR4 recruits TRIF adaptors. Interestingly, when TRIF recruits RIP1 protein kinase it activates the very same steps as in MyD88 dependent pathway, when it activates PI3K, it induces MAPK pathway and finally when it activates TRAF3 it activates IRF3 and IRF7 [10].

5.3.2 Direct activation of TLRs in $CD4^+$ T cells

Studies characterizing TLRs function have been mainly focus on the cells of innate immunity, but there is evidence that TLRs are important for T cells function and adaptive immunity as well. At the mRNA level, expression of all TLRs was confirmed for CD4+ T cells [24-26]. Expression of TLR2, 3, 4, 5, 9 has been also detected by flow cytometry [27-29]. Since TLRs expression in T cells can differ in naive, memory and regulatory cells, it is suggested that it

depends on their functional and activation status [30-34]. Expression of selected TLRs and its effect on T cells is shown in Table 2.

The most of the knowledge about the role of TLRs in T helper cells come from studies about TLR2. Toll-like receptor 2 signalling in naive CD4+T cells promotes IFN-γ production and differentiation into Th1 cells. Furthermore, TLR2 is the only TLR, which could induce proliferation and IFN-γ production from already differentiated Th1 cells [28]. Additionally, TLR2 influence Th17 cells development and function since Th17 cells differentiation was found to be promoted by TLR2 [35, 36]. Moreover, restimulation of activated T cells with TLR2L induced production of IL-17 [37]. In a Th17 dependent EAE (experimental autoimmune encephalomyelitis) model, T cells lacking TLR2 were unable to induce the disease [36]. In summary, TLR2 activation is involved in the regulation of Th1 and Th17 cells functions.

On the contrary, proliferation and survival of the naive T cells activated via TLR4 is observed, rather than the direct promotion of T helper cells differentiation [38]. *In vivo,* the role of TLR4 in T cells highly depends on the site of action. In the central nervous system (CNS), loss of TLR4 in CD4+T cells, protected against EAE via decreased IFN-γ and IL-17 production at the site of infection but not in the periphery [38]. On the contrary, in gut TLR4 deletion led to enhance IFN-γ production and severe colitis [39]. Finally, activation of TLR4 on T cells enhances the ability of CD4+T cells to bind fibronectin and migrate [40].

Growing number of evidence suggest that not only plasma membrane bound TLRs are functional in CD4+T cells but also activation of human CD4+T cells via endosomal TLR9 enhanced expression of activation markers and entry into the cell cycle [41]. Unlike for TLR2, 4 and 9, most studies about TLR3 function in T cells were performed in humans. TLR3 is expressed on human CD4+ and CD8+ T cells [41] and its stimulation induces the transcription factor IRF7 [42], NF-kB and MAPK, survival of CD4+T cells [43] and CXCL10,

CCL3, CCL4, CCL5 expression [42]. Interestingly, stimulation of TLR3 with poli (I:C) in the presence of anti-CD3 and anti-CD28 antibodies, induced NF-kB dependent synthesis of IL-17A and IL-21. Stimulated cells do not express classical helper T cell transcription factors like T-bet, Gata-3 nor RORγt [44].

Several groups have shown that in the absence of CD28 involvement, TLRs may provide co-stimulatory signals for T cells [27, 28]. Importantly, in the absence of TCR stimulation, TLRs do not induce functional responses in naive T cells, preventing non-specific T cell activation by TLRs alone [45].

Table 2. Effect of selected TLRs on activation of T cells

TLRs	Expression in T cell subsets			Effect on T cells
	Naïve	Activated/Memory	iTreg	
TLR1	±	++	+	⇒ Increased proliferation and survival ⇒ Abrogate the suppressive function of Tregs
TLR2	±	++	+	⇒ Increased cell proliferation and survival ⇒ Promote cytotoxic activity of CTL ⇒ Generate efficient memory T cells ⇒ Augment Treg cell proliferation with temporal loss of suppression ⇒ Induction of cytokines production (IFN-γ, IL-17A, IL-10, IL-13, CCL5, CXCL10)
TLR3	+	++	-	⇒ Promotion of activated CD4+T cells survival ⇒ Induction of cytokines (CXCL10, CCL3, CCL4, CCL5, IL-17A, IL-21)
TLR4	±	++	+	⇒ Induce Treg cell activation ⇒ Enhance the suppressive function of Tregs
TLR6	+	+	+	⇒ Block the suppressive function of Tregs
TLR9	+	++	-	⇒ Promote survival of activated CD4+T cells ⇒ Inhibit Treg cell suppression

Expression levels: (-) not detectable, (±) weak or low expression, (+) normal, (++) enhanced

5.4 The adaptive immune system

T and B lymphocytes are the essential cells of the adaptive immune system. In contrast to cells of the innate immunity, mature B and T lymphocytes differ from each other in terms of specificity – they express a highly specific and diverse repertoire of antigen recognizing receptors on the cell surface. The adaptive branch of the immune system may be subdivided in to humoral and cellular-mediated immunity, which differ in the mechanisms they use and the type of pathogens they respond to. Antibodies, the main molecules of humoral adaptive immune system, are involved in microbial antigen recognition, and elimination in blood and mucosa tissues. In general, this type of immunity targets extracellular microbes and their products by direct binding with produced antibodies. On the other hand, cellular immunity involves T helper cells for the induction of immunity against intracellular microbes that are normally not accessible for antibodies. This section focuses on the T cell mediated immune responses, with a special emphasis on CD4+ T helper cells.

5.4.1 T lymphocytes

5.4.1.1 T cell receptor (TCR)

T cells recognize antigens that are presented via major histocompatibility molecules (MHC) by antigen presenting cells (APC). As a result, T cells do not respond to soluble antigens but only to cell-surface associated antigens. The TCR was discovered in the early 1980s, using monoclonal antibodies recognizing a single cloned T-cell line [46]. There are two types of TCRs, both heterodimers, both consisting of transmembrane polypeptide chains. Most of the circulating T cells express αβ while the minority expresses γδ chains. Each chain of the TCR consists of a short cytoplasmatic region, hydrophobic transmembrane region, N-terminal variable domain (V) and constant domain (C). In variable domains of the two chains three hyper-variable, so-called complementarity-determining regions (CDRs), are present. CDRs are the part of TCRs that specifically recognize peptide-MHC complexes [47]. The

cytoplasmic regions of TCRs are too short to transduce signals into the cell and trigger activation. Signaling via TCR depends on a complex of CD3 molecules: δε (delta/epsilon), γε (gamma/delta), ζζ (zeta/zeta) dimers. Cytoplasmic parts of all CD3 molecules contain immune-receptor tyrosine-based activation motifs (ITAMs) [48]. During the antigen recognition by T cells, ITAMs become phosphorylated and initiate signal transduction. The contribution of the CD3 complex in the signal transduction by TCR is not sufficient to activate the cells. To facilitate signaling and T cell activation, CD4 and CD8, two TCR co-stimulatory molecules, recognizing the MHC-peptide complex, are necessary. CD8+ T cells recognize peptides presented by antigen presenting cell in the context of MHC class I and function primarily as cytotoxic cells, while CD4+ T cells recognize MHC class II molecules and function mainly as helper cells [49, 50]. Figure 1 shows the structure of the TCR receptor complex.

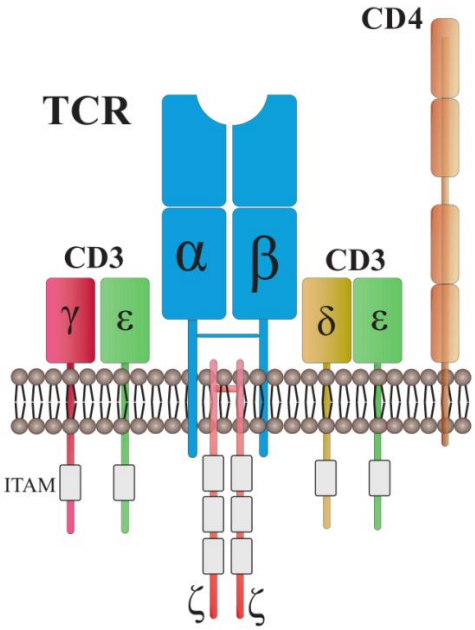


Figure 1. T cell receptor complex on CD4+ T cells.

5.4.1.2 TCR receptor gene rearrangement

Microorganisms rapidly evolve and to protect the host, the immune system must be diverse and flexible in their recognition. B and T cells express highly diverse antigen receptors. The diversity of receptors are generated during the development of B and T cells. In this section, the principles of TCR gene rearrangement are described.

Each TCR locus consists of V and J gene segments, as well as C region located 3` of J segment. There are 2 constant region genes in each of human TCR β and TCR γ and only one each for TCR α and TCR δ. For all TCRs, the constant region is composed of 4 exons encoding: the cytoplasmic tail, the transmembrane segment, the hinge region, and the extracellular region. In addition TCR β and δ loci have D segments. During the T cell development in the process of somatic recombination a functional V exon region of the α chain is form when the Vα segment rearranges to the Jα gene segment. Transcription and splicing of the VJα exon to Cα generates the mRNA that is translated to TCRs chain protein. In the case of the β chain, the rearrangement of Vβ, Dβ, Jβ segments generates a functional VDJβ V-region exon that is transcribed and spliced to join Cβ. Finally, mRNA is translated to T-cell receptor β chain, and soon after synthesis the α and β chains form a heterodimer [51, 52]. The combinatorial and junctional diversity during the process of gene rearrangement shape the diversity of T cell receptors. Lastly, T cell receptors after completing rearrangement do not diversify further their variable regions in the process of somatic hypermutation as it happens in case of antibodies [2].

5.4.2 Antigen presentation and T cell activation

In contrast to B cells, T cells respond only to short amino acid sequences that are formed when protein is unfolded, processed and cut into peptides, which displayed in the context of major histocompatibility molecules [53, 54]. There are 2 types of MHC molecules, which differ in structure, the nature of presented antigens and expression patterns. Distinct distribution of MHC class I and class

II molecules in cell types triggers different effector functions by T cells. MHC I is expressed on almost all nucleated cells and presents peptides derived from intracellular pathogens. MHC I present peptides to CD8 cytotoxic T cells, which in turn kills the infected cells. On the contrary, CD4 T cells recognize MHC II-peptide complexes and activate other cells of immune system. MHC II molecules are expressed exclusively on professional APC`s: DC`s, macrophages and B cells. If antigen is presented by B cells to CD4+ T cells, they stimulate B cells to produce antibodies. When foreign antigen is presented by macrophages, T cells activate them to destroy the pathogens inside the cell [55-57]. From all professional APC`s, DC`s are considered as the most effective in the antigen presentation to naive T cells [58, 59]. If the pathogen enters body directly into the bloodstream it is taken up by APCs located in spleen. Pathogens infecting tissues directly, are recognized by immature tissue resident DC`s. During the antigen (Ag) recognition DCs are activated by PAMP, DAMPs or via cytokines produced during the inflammatory response. Next, activated DCs migrate to the nearest lymph nodes, where they present antigen to antigen specific naive T cells, activate them and trigger differentiation into effector T cells [60, 61].

T cell development occurs in the thymus, where T cells with diverse TCR specificities, tolerant to self and restricted to self-MHC molecules are generated. As soon as T cells undergo positive and negative selection and their lineage is committed, they leave the thymus as a mature, naïve T cell enter the circulation [62-65]. Later, they re-circulate between the blood and peripheral lymphoid organs, searching for specific peptides presented via MHC molecules on APC. In lymph node, naive T cells binds temporally to every APC via LFA-1, CD2 and ICAM-1, ICAM-2, CD58 interaction between T cells and APCs. At the time, when naive T cell recognizes specific peptide loaded MHC molecules, the initial binding of adhesion molecules increases its affinity and the connection between APC and naive T cell is stabilized. This allows the formation of so-

called immunological synapse or supramolecular activation complex (SMAC). During formation of immunological synapse, the contact surface between APC and T cell is organized into 2 areas: the central supramolecular activation complex (c-SMAC), where MHC-TCR, co-stimulatory and co-receptor molecules, responsible for T cell activation are located, and the peripheral-SMAC (p-SMAC) where adhesion molecules and their ligands, cytoskeletal protein connecting integrins to actin are found. Clustering of T-cell receptors in the c-SMAC triggers re-orientation of cytoskeleton and polarization of T cell towards the APC [66-68]. Signal provided only by the recognition of peptide bound to the MHC molecule by the TCR on T cells is not enough to trigger T cell activation and differentiation [69]. Two other signals are indispensable: interaction between costimulatory molecules that provide the 2^{nd} signal, and cytokines produced by APCs that directly drive T cells differentiation into specific effector subsets, serves as the 3^{rd} signal. CD28 is the best characterized costimulatory receptor of T cells that binds to CD80 or CD86 costimulatory ligands on dendritic cells to administer signal number 2 [70, 71]. CD28 is expressed by all naïve and activated human CD4+T cells The main role of CD28 – CD80/CD86 interaction is to enhance TCR induced proliferation and survival by inducing expression of IL-2 and α chain of IL-2 receptor by T cells [72]. T cells activated in this way divide rapidly to generate a sufficient number of antigen specific T cells to eliminate antigens in process called clonal expansion. The 3^{rd} signal, is provided by polarizing cytokines, which determines the functional fate of activated T cells. CD4+T cells differentiate into effector cells that are characterized by: subset inducing polarizing cytokines, lineage specific transcription factors, and a range of cytokine produced to convey effector function [73]. The main classes of effector T cells are: Th1, Th2, Th9, Th17 and Th22 cells [74, 75]. Figure 2 shows the immunological synapse formation between antigen presenting cell and T cell.

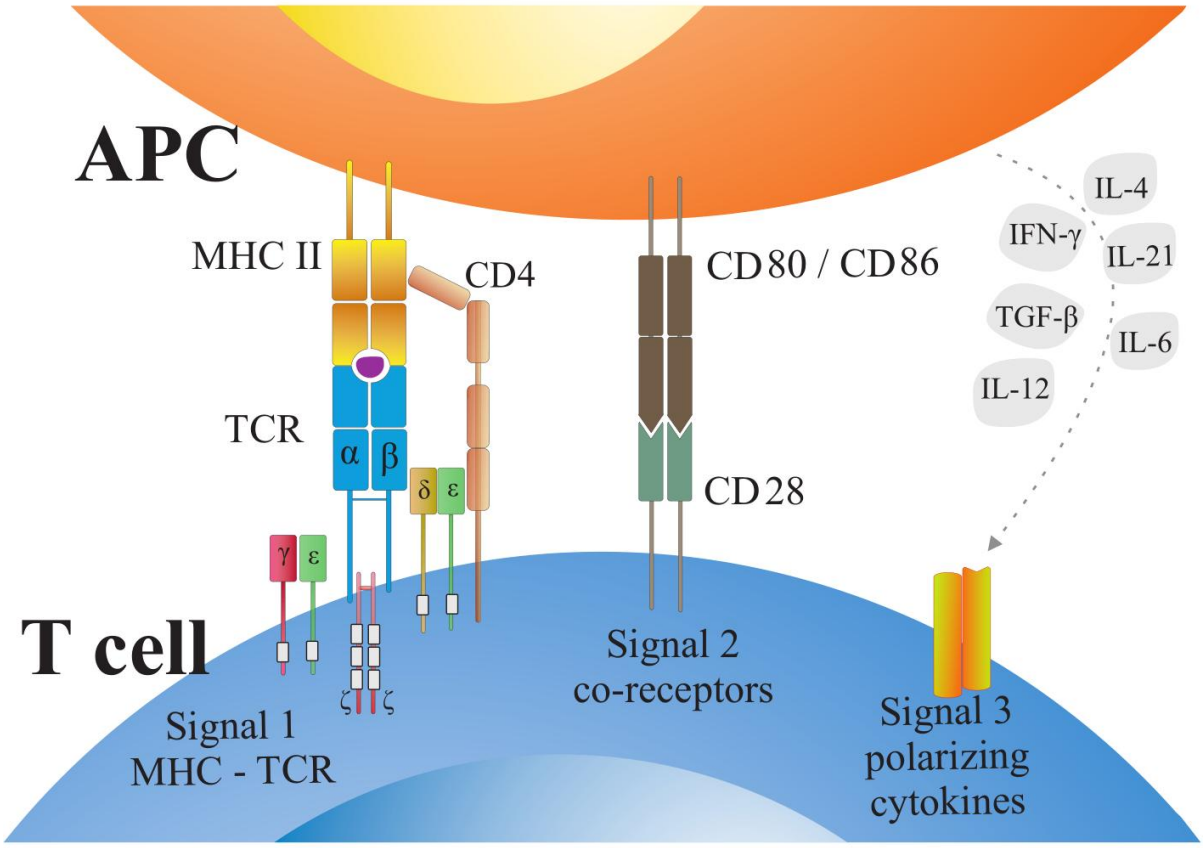


Figure 2. The immunological synapse formation and 3 signals activating T cells

5.4.3 Effector T helper cell subsets

5.4.3.1 Th1 cells

IFN-γ and IL-12 are the 3rd signal cytokines that when provided to naïve T cells drive their differentiation into Th1 cells [76]. Those cytokines are produced in response to microbes by activated DC`s, macrophages and NK cells. Two signal-transducing activators of transcription molecules (STATs), STAT 1 and STAT 4 contribute to Th1 cell development and are activated by IFN-γ and IL-12, respectively. Activated STAT 1 induces expression of T-bet transcription factor, the master regulator of Th1 differentiation [77]. The main function of T-bet is activation of gene transcription of IFN-γ and IL-12 receptors and IFN-γ. Promotion of the expansion and differentiation of Th1 cells is provided by IL-12 activated - STAT4 that enhances further IFN-γ production. In the process of cross-regulation between other T helper subsets, differentiation of naïve T cells to Th2 and Th17 cells is inhibited by IFN-γ. The main function of Th1 cells is the protection against intracellular pathogens. IFN-γ produced by Th1 cells, activates macrophages to neutralize phagocytosed pathogens [78]. Additionally, IFN-γ promotes antibody class switching mostly to IgG isotypes. These antibodies help complement activation and phagocytoses of opsonized microbes [79]. Recent work from our group showed the relationship between Th1 mediated immune responses and epithelial tissues. Upon IFN-γ stimulation of epithelial cells from patients suffering from chronic rhinosinusitis downregulation of tight junction proteins and defects in epithelial cells were observed [80]. In skin, IFN-γ increased the apoptosis of keratinocytes [81].

5.4.3.2 Th2 cells

The development of Th2 cells from naïve T cells is driven by IL-4 [78, 82]. Since DC`s can`t produce IL-4, the main source of this cytokine are eosinophils, basophils, mast cells, NKT cells and germinal center B cells as well as T follicular helper cells (Tfh cells). As a consequence of IL-4 binding to

its receptor on naive T cells, STAT6 is activated. Next, STAT6 promotes GATA3 expression, which is the master transcription factor of Th2 cells and activates the genes for the hallmark cytokines produced by Th2 cells: IL-4, IL-5 and IL-13 [83]. Additionally, GATA3 stabilizes Th2 differentiation from naïve T cells by inducing its own expression. Th2 cytokines play major role in host defense against helminths. IL-4 and IL-13 are indispensable cytokines for immunoglobulin heavy chain class switch into IgE antibodies. Additionally, another Th2-related cytokine, IL-5 activates eosinophils and induces their differentiation and migration. Eosinophils express receptors for IgE and when the receptor is cross-linked by IgE Ab bound to the parasite, inflammatory mediators are released. Moreover, IL-4 and IL-13 contributes to alternative macrophages activation, stimulate peristalsis in the gastrointestinal track, increases mucin secretion from gut and airway epithelial cells, intestinal epithelial cells turnover, smooth muscle contraction [83-87], that collectively helps to facilitate the removal of parasites from the gut. On the other hand overproduction of type 2 cytokines may have detrimental effect mainly on allergic disorders like asthma, allergic rhinitis, atopic dermatitis, allergies to drugs and food [88]. Additionally, our recent data showed that IL-4 and IL-13 disrupt epithelial cells tight junctions and might be responsible for impaired, leaky epithelial barrier in asthma (data not published).

5.4.3.3 Th17 cells

As soon as naïve T cells are activated by IL-6, TGF-β and IL-23 their differentiation is directed into Th17 cells [89-91]. Polarizing cytokines activate STAT3 transcription factor, which further induce orphan steroid receptor RORγt, a key transcriptional regulator of the Th17 cell fate [90, 92, 93]. The name of Th17 cells comes from the fact that the main cytokine produced by this subset is IL-17A and IL-17F. Furthermore, Th17 cells may produce IL-22 that is responsible for epithelial cells homeostasis and antimicrobial defense [94], along

with TNF and GM-CSF, which provide the activation and recruitment of neutrophils [95]. Expansion of Th17 cells is promoted by IL-23 and Th17 cells are notably abundant in mucosal tissues. The main function of Th17 cells is induction of neutrophilic inflammation that recruits neutrophils to ingest and kill extracellular microbes. Nevertheless, pathogenesis of psoriasis, inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis is associated with Th17 cells [96].

5.4.3.4 Th9 cells

Initially, IL-9 was characterized as a Th2 cell derived cytokine. Later, a separate Th9 subset of cells was defined. The differentiation of Th9 cells occurs in the presence of a signal delivered by TGF-β and IL-4 [97-99]. Interestingly, neither TGF-β nor IL-4 itself is sufficient to induce the Th9 profile with high levels of IL-9. Development and maintenance of Th9 cells depends of IRF4 and PU.1, but so far, the master regulator of transcription has not been found [100, 101]. Both, protective as well as pro-inflammatory role for Th9 cells in mouse models and humans has been described. Protective immunity in infection with the parasitic worm *N. brasiliensis* [102], and in inhibition of subcutaneous [103] and pulmonary [104] melanoma growth was mediated by Th9 and Th9-derived IL-9. On the contrary, in EAE mouse model, IL-9 attracted pathogenic Th17 cells to the central nervous system via the induction of CCL20 chemokine expression in astrocytes and induced EAE symptoms [105]. In the ovalbumin-induced model of airway inflammation, IL-9 neutralization strongly reduced allergic symptoms [100] and the transfer of OVA-specific Th9 cells into T-cell deficient mice caused asthmatic symptoms [101]. In humans, increased numbers of Th9 cells has been shown in the blood of allergic patients [106]. In summary, protective as well as inflammatory role for Th9 cells in mouse models and humans have been described. Figure 3 shows the main subsets of T helper cells with their key transcription factors and hallmark cytokines.

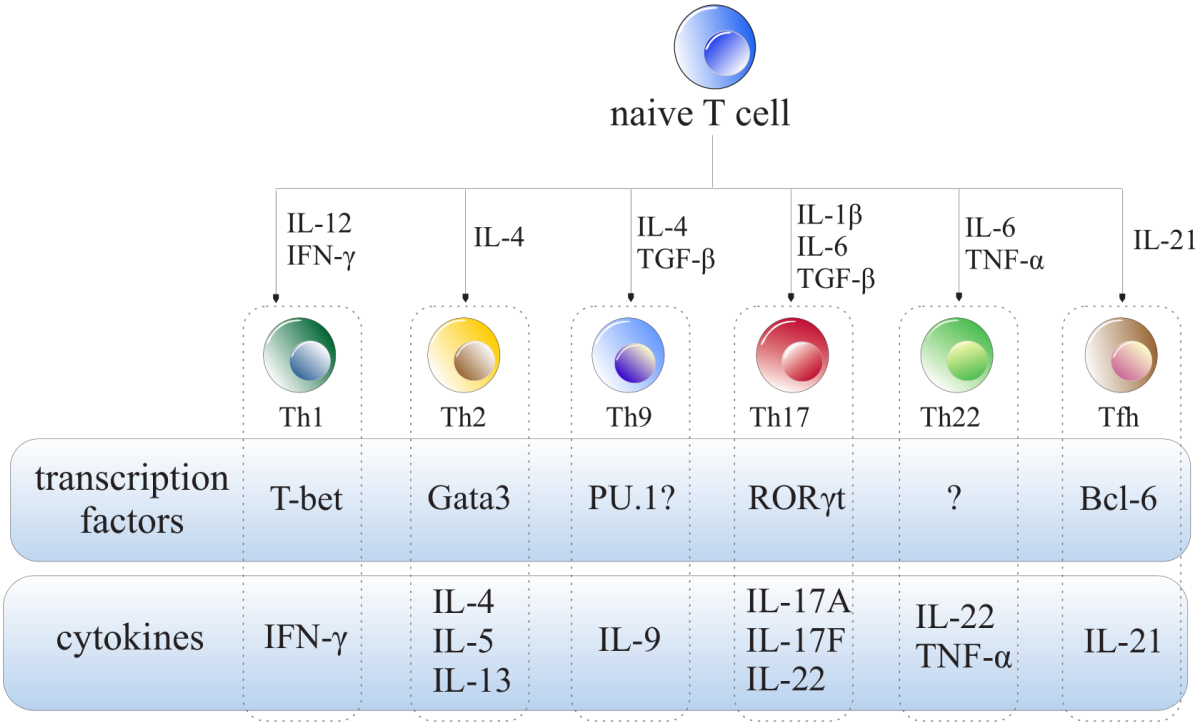


Figure 3. Subsets of T helper cells (Adapted from [107])

5.4.3.5 Th22 cells

5.4.3.5.1 IL-22 – gene, structure and signalling

Interleukin-22 was discovered in 2000 as the "IL-10-related-T-cell-derived inducible factor (IL-TIF) in murine IL-9 stimulated BW5147 T lymphoma cells and discovered in human cells the same year [108-110]. IL-22 together with IL-19, IL-20, IL-24 and IL-26 are members of IL-20 subfamily of cytokines, which along with IL-10, IL-28A, IL-28B and IL-29 is a part of larger IL-10 cytokine family [111]. Human IL-22 gene is located at chromosome 12q15 in the proximity of the genes encoding IFN-γ and IL-26 [109]. The other members of IL-20 subfamily, (IL-19, IL-20, IL-24) are located on chromosome 1q32. Mouse and human IL-22, a 179-amino acid protein, shares 79% homology and is encoded by a 537-base pair open reading frame [108]. Additionally, murine IL-22 shares 22% and human IL-22 shares 25% sequence identity with IL-10

[108]. After removal of 33-amino acid signal peptide, a 146-amino acid protein in the active form is secreted [112, 113]. IL-22 is a monomer that may form non-covalently bound dimers and if present at high concentration tetramer are formed [113, 114]. As a monomer, IL-22 has a bundle-like structure that is composed of six α-helices and a small N-terminal helix stabilized by two intramolecular disulphide bridge bonds. Despite the fact that glycosylation has only a small effect on IL-22 tertiary structure, IL-22 has 3 potential glycosylation sites [113, 115].

When produced, IL-22 binds to IL-22R, which consists of two subunits, IL-10R2 and IL-22R1 [116, 117]. IL-22R is a type 2 cytokine receptor with the typical structure consisting of an intracellular, transmembrane and extracellular moiety. STAT recruitment sites are located in the intracellular moiety of IL-22R1 rather than IL-10R2 [117]. The binding of IL-22 to IL-22 receptor is two-step process. In the first step IL-22 binds to the extracellular moiety of IL-22R1 as it has high affinity for IL-22R1 (K_d = 1-20nM) but no affinity to IL-10R2. The initial binding of IL-22 to IL-22R1 changes the conformation of the cytokine. In the next step IL-22/IL-22R1 complex binds to IL-10R2 (K_d=7-45uM), which enables downstream signalling [116, 118-120]. Interestingly, both subunits of the IL-22 receptor may be used by other members of IL-10 cytokine family. IL-10R2 is a part of IL-10 receptor (IL-10R1/IL-10R2), IL-26 receptor (IL-20R1/IL-10R2), IL-28 and IL-29 receptor (IL-28R1/IL-10R2) [121]. Meanwhile, the second IL-22 receptor subunit, IL-22R1, is used by IL-20 and IL-24 (IL-20R2/IL-22R1) and may mediate signalling and trigger effects similar to IL-22R [122, 123].

5.4.3.5.2 IL-22 receptor

The binding of IL-22 to IL-22R1/IL-10R2 complex induces activation and phosphorylation of Jak1/Tyk2 tyrosine kinases that then phosphorylates tyrosine residues in the intracellular part of IL-22R1. Phosphorylated tyrosine residues of

IL-22R1 attract STAT molecules, which are later phosphorylated by Jak1/Tyk2 kinases [124]. Phosphorylated STAT3 is the key mediator of IL-22 function, however phosphorylation of STAT1 and STAT5 have also been reported. In addition to STAT mediated signalling IL-22 stimulation activates MAPK and PI3K-AKT-mTOR pathways as well [125].

It is well established that IL-22 is unique cytokine, which is produced by immune cells, acting solely on non-immune tissue cells. Cell sensitivity to IL-22 is determined by expression of IL-22R1 subunit. The IL-10R2 subunit is expressed commonly in all cells, however IL-22R1 subunit is not detected on immune cells (monocytes, monocyte-derived macrophages, DCs, B cells, T cells, NK cells), but mainly on cells of epithelial origin in skin, small intestine, colon, liver, kidney, pancreas and lung [126, 127].

Another level of regulation of IL-22 function is determined by existence extracellular IL-22-binding protein (IL-22BP). IL-22BP can be detected in normal tissues like thymus, spleen, lymph node, stomach, intestine, lungs, skin, placenta and breast [128-131]. The main cell subsets producing IL-22BP are immature dendritic cells. The production of IL-22BP by DCs decreases as soon as DC become mature [132, 133]. IL-22BP is a soluble, 210-amino-acid-long, secreted, single-chain protein that is encoded by the IL-22R1-independent gene [129, 131, 134, 135]. IL-22BP has 20-1000 times higher affinity to IL-22 than IL-22R1 [120]. Moreover, the part of IL-22 involved in binding with IL-22BP overlaps with the part of IL-22 that is engaged in binding to IL-22R1 [136, 137]. Taken together, IL-22BP has an inhibitory action towards IL-22 action by preventing the binding of IL-22 to IL-22R1/IL-10R2 complex.

5.4.3.5.3 IL-22-producing cells

The main producers of IL-22 are T cells and innate lymphoid cells (ILC`s). The first subset of T cells reported to produce IL-22 were Th1 cells [126]. Among all IL-22-producing human T helper cells in peripheral blood, up

to 35% are Th1 cells [138]. IL-22 and IFN-γ are localized closely on the 12th chromosome and it has been shown that IL-22 production correlates the best with IFN- γ production and T-bet expression [139]. Similarly, in mouse models, Th1 cells may produce IL-22 and the production can be upregulated by IL-12 and synergistically acting with IL-18 [140].

IL-22 is also secreted by Th17 cells. Most of controversy in the assigning IL-22 as Th17 rather than Th22 cytokine comes from discrepancy between mouse and human data. Among all mouse T helper subsets, Th17 cells are the major source of IL-22 and IL-22 is named as Th17 cytokine [141-143]. On the contrary, IL-22 production by human T cells does not correlate with either RORγT or IL-17A [139] and only 10-18% of IL-22-producing T cells co-express IL-17A in the blood [138]. IL-1β, IL-6, TGF-β and IL-23, Th17 polarizing cytokines differently influence IL-22 production by Th17 cells. *In vitro* stimulation of naive T cell with IL-6 alone, induces IL-22-single producing T cells, while the combination of IL-1β, IL-6 and IL-23 induces Th17 cells co-expressing both, IL-17 and IL-22. On the contrary, IL-6 combined with TGF-β generate Th17 cells with little or no IL-22 production [144]. In summary, despite TGF-β, all Th17 polarizing cytokines promote IL-22 production. Indeed, it has been shown that TGF-β inhibits IL-22 production from murine Th17 cells through the c-Maf dependent mechanism [145].

In human, the IL-22-only-producing T helper cells have been identified in peripheral blood mononuclear cells (PBMC`s) and defined as a separate T helper subset named Th22 cells [138, 146, 147]. 37% - 68% of all human IL-22-producing T cells in peripheral blood do not co-produce IFN-γ nor IL-17A [138]. Despite the fact that, similar cells were reported in mice, the IL-22-only-producing mice T cells have not been separated and defined as Th22 cells [148]. The common feature of Th17 cells and Th22 cells is that they share a requirement for IL-6. In the case of Th22 cells, TCR stimulation combined with IL-6 and TNF-α prime Th22 development from naïve T cells [138].

Additionally, epidermal Langerhans cells, dermal DC`s, plasmacytoid DC`s promote the induction of Th22 cells [138, 149]. The Th22 cells express the pattern of chemokine receptors, mostly directing them to skin – CCR6, CCR4 and CCR10, but do not express CXCR3 [138, 146, 147]. To some extent, Th22 cells may have an autoreactive phenotype and react to auto-glycolipids presented on CD1a by Langerhans cells [150]. One of the possible reasons why IL-22-producing T cells are not completely defined as a separate Th22 subset is due to the lack of a key transcription factor responsible for induction of IL-22 production. A promising candidate to fulfill this role is aryl hydrocarbon receptor (AhR). AhR is specifically expressed by Th17 cells, however AhR-deficient T cells still differentiate into Th17 cells that lack IL-22 production. Nevertheless, AhR seems to be transcription factor that promotes IL-22 production from Th17 cells rather than a bona fide Th22 cells transcription factor. Further experiments have to be performed to completely understand the role of AhR induction of IL-22 in Th22 cells [151].

Th1, Th17 and Th22 cells are not the only cells producing IL-22. As in case of CD4+ T cells, CD8+ T cells may produce either IL-17/IL-22 (Tc17) or IL-22 alone (Tc22). Human Tc22 cells differentiate from naïve cells upon IL-21 stimulation [152] and similar to Th22 cells, TGF-β suppresses IL-22 and induces IL-17A production [153]. Identical Tc17 populations capable of producing IL-17 and IL-22 have been described in mouse models [154, 155]. Cells from the innate arm of immunity like γδ T cells, NKT cells and ILC3 cells may also produce IL-22. In case of γδ T cells, IL-22-producing cells express RORγt and IL-22 production is triggered in response to IL-23 and TLR`s ligands [156-158]. NKT cells have been reported also to produce IL-22 [159, 160]. NKT-IL-22-producing cells just like αβ Tcells, γδ T cells and ILC3s express RORγt, IL-23R and CCR6. Further, IL-22 production by NKT cells, requires antigen presentation via CD1a molecules on antigen presenting cells [161].

It has been shown that in the *Rag2-/- deficient* murine gastrointestinal model of infection with *Citrobacter rodentium*, the level of IL-22 remain unchanged, despite the lack of all T and B cells [162]. In mouse models of infection, at the later stages of infection Th22 cells are the main source of IL-22 while during the early phases ILC3s are the main producers of IL-22. After years of research, it turns out that the dominant source of IL-22 in the gastrointestinal track are ILC`s group 3 [163-166].

Recently, it has been described that neutrophils contribute to antimicrobial defense and reconstitute epithelial integrity during colitis by IL-22 production [167]. Surprisingly, Mashiko et al. reported that mast cells are the major IL-22-producing cells found in patients with psoriasis and atopic dermatitis [168].

5.4.3.5.4 Effect of IL-22 on tissue cells

In general the effect of IL-22 on epithelial cells in the gut, skin and lungs can be divided into five main effects. First of all IL-22 increases innate defense mechanisms against microbes via the induction of antibacterial proteins (β-defensins, members of S100A family, regenerating islet-derived protein (REG) family members) and mucus associated proteins [162, 169-171]. Secondly, especially in the skin, IL-22 influences keratinocytes differentiation by reducing the expression of keratins, profilaggrins, kallikreins, desmocollins, proteins, which are necessary for keratinocytes terminal differentiation [171, 172]. Similar effects can be observed in epithelial cells from the gut and respiratory track where IL-22 inhibits cell differentiation but at the same time enhances their proliferation [173]. Furthermore, production of chemokines, mostly neutrophil attracting chemokines like CXCL1, 2, 5, 8 are increased in keratinocytes stimulated with IL-22 [169]. In addition, increased expression of extracellular matrix degrading enzymes (Matrix metalloproteinase 1 and 3 - MMP1 and MMP3) was promoted by IL-22 and by the enhanced migratory ability of epithelial cells [171, 172]. Finally, IL-22 amplify its own action by

positive feedback loop and increased expression of IL-20, which binds to IL-22R1/IL-20R2, and increase expression of STAT3, main molecule of intracellular signal downstream of IL-22R [174, 175], (Figure 4.) On the contrary, when uncontrolled, IL-22 primary helpful function may lead to pathology. In the next chapter the main pathological functions of IL-22 in different diseases will be discussed.

Psoriasis

Psoriasis is the first immune mediated disorder that has been associated with inflammatory IL-22 function [127]. IL-22 expression is upregulated in lesional skin as compared to non-lesional psoriatic and healthy skin [127]. The anti-psoriatic therapy significantly decreases IL-22 expression in lesional skin [171, 176]. Among all IL-22-producing cells in psoriasis the majority are dermal CD4+ T cells including Th22 and Th17 and to lesser extent Th1 cells [177]. On the contrary, in the epidermis the number of Tc22 that do not produce IFN-γ and IL-17 was also increased [178]. On the contrary to IFN- γ and IL-17, cytokines that are highly expressed in lesional skin, only levels of IL-22 increases in blood of psoriatic patients and correlate with disease severity [171]. The pathological influence of IL-22 on keratinocytes includes, epidermal hyperplasia caused by altered terminal differentiation, secretion of granulocyte chemoatracting agents that accumulate neutrophils in psoriatic lesion and finally, induction of IL-22 production and STAT3 expression [172, 175]. Interestingly, despite the dysfunctional epithelial barrier, psoriatic skin is resistant to bacterial infection, mainly due to the induction of anti-microbial peptides production by IL-22 stimulated keratinocytes [171]. The effect of IL-22 on keratinocytes differentiation is IL-22-specific and neither IL-17 nor IFN-γ influence or amplify this action [175, 179]. However, IL-17 and IFN-γ may only strengthen IL-22 induced production of antimicrobial peptides, cytokines and chemokines [175]. Finally, in the epidermis of psoriatic skin expression of IL-22R is

increased. Cytokines produced locally in psoriatic skin, mainly IFN-α and TNF-α, additionally upregulated IL-22R1 expression and amplify the effect of IL-22 [175, 180, 181].

Atopic dermatitis

IL-22 is also elevated in the inflamed skin of atopic dermatitis (AD) patients [127]. Compared to psoriasis, the main producers of IL-22 in AD are Th22 and Tc22 cells capable of co-expressing IL-13 [177, 182]. The chronic form of AD is characterized by the conversion of immune responses from Th2-mediated to Th1/Th2/TH22 mixed responses. Often, patients with AD suffer from cutaneous infections since other cytokines, like IL-17 are not expressed and cannot cooperate with IL-22 in the induction of antimicrobial peptides. Levels of IL-22 in the serum correlates with the expression of CCL17, a chemokine that attracts T cells expressing CCR4 to skin of AD patients [183].

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is chronic inflammatory disease of gastrointestinal track that is subdivided into 2 forms: ulcerative colitis (UC) and Crohn`s disease (CD). In inflamed intestines of both forms of IBD, increased expression of IL-22 is detected [184]. In patients with UC, IL-22-producing T helper cells are mostly located within the lamina propria, while in CD patients Th22 cells are detected throughout the intestinal wall, and higher numbers of Th22 cells are present in CD as compared to UC patients [184]. Additionally to local tissue expression, IL-22 is detected in the blood of CD patients and correlate with disease severity [185]. IL-22 is considered to play a protective role in IBD by acting on intestinal epithelial cells and inducing: production of antimicrobial peptides (Reg family of c-type lectins, defensins, cathelicidins) that modulate the colonic microbiota [186], expression of mucus-associated

molecules and reconstitution of the mucus-producing goblet cells [187], epithelial cells proliferation that contributes to the regeneration and repair of epithelial layers [188]. It should be taken into account that despite this protective role of IL-22 in IBD, amplification of intestinal inflammation by IL-22 was reported in a colitis model induced by transferring $CD45Rb^{low}$ CD4+ memory IL-10-deficient T cells [189].

Lung inflammation and asthma

An increased levels of IL-22 mRNA and protein have been reported in the blood of asthmatic patients and in murine asthma models [190, 191]. IL-22 has a dual role in asthma-associated lung inflammation [191]. When administrated during allergen challenge, IL-22 reduced the expression of Th2-derived cytokines and chemokines, eosinophils infiltration and airway constriction. Consequently, neutralization of IL-22 exacerbated lung inflammation and pathology. On the contrary, the role of IL-22 during the sensitization phase depends on the route the allergen was applied. Subcutaneous sensitization of IL-22 exhibits a pathologic role and exacerbated lung inflammation as compared to intraperitoneal sensitization [192-194]. Inhibition of CCL17 and IL-25 production, minimizes lung cell damage and the possible involvement of IL-10 could play a potential role in the dual function of IL-22 in lungs [192, 194].

Additionally, the lung is an organ where the role of IL-22 depends on the other cytokines present in the inflamed airways. In the model of bleomycin-induced fibrosis, in the presence of IL-17A, IL-22 contributes to airway inflammation while in the absence of IL-17, IL-22 exhibits a tissue protective role [195].

Rheumatoid arthritis

In general IL-22 targets only epithelial cells, however synovial tissue fibroblasts have been also described as expressing IL-22R1 [175]. In the

synovial tissues and cells from the synovial fluid of patients with rheumatoid arthritis (RA), high levels of IL-22 mRNA were reported [196]. IL-22 is increased not only in synovial tissue but also in the serum of patients with RA, where the increased number of Th22 cells can be observed [197-199]. Levels of IL-22 in serum correlated with disease severity and progression [197, 199]. By inducing proliferation of synovial fibroblasts, IL-22 contributes to the pathology of RA [196]. Moreover, IL-22 stimulated fibroblasts produce CCL2 and RANKL production from fibroblasts, which attract monocytes to the synovium and induces their differentiation to osteoclasts, respectively [196, 200].

Hepatitis

Hepatocytes are the main targets of IL-22 in the liver. The protective role of IL-22 in liver inflammation was shown in mouse models, where IL-22 prevented: 1) concavalin-A induced liver injury, necrosis and apoptosis via IL-22-dependent STAT3 activation and induction of anti-apoptotic proteins [201], 2) hepatocyte necrosis during *Salmonella* infection [202] and general liver pathology and lethality in malaria infection [203], 3) liver ischemia-reperfusion injury [204], 4) acute as well as chronic alcohol induced liver damage [205]. In general, IL-22 action in liver mainly relies on: induction of acute-phase proteins (Amyloid A, α1-antichymotrypsin, haptoglobin, LPS-binding protein) and anti-apoptotic proteins production from hepatocytes [127, 185, 206] and promotion of liver stem/progenitor cells proliferation [207].

Pancreatitis

The pancreas is the organ where tissue expression of IL-22R1 is the highest among all organs [127]. Within the pancreas, IL-22R1 is expressed by acinar and islet cells [208, 209]. The therapeutic potential of IL-22 in pancreatitis has been suggested [210-212]. The main protective effect was linked to IL-22 induced production of REG3β and REG3γ molecules and Bcl-2

and Bcl-X_L anti-apoptotic proteins by acinar cells. Additionally, islet cells may produce Reg1 and Reg2 molecules and increase proliferation upon IL-22 stimulation, which may contribute to overall protective effect of IL-22 in pancreatitis [210-212].

Host defence against microbial infection

The initial work of Graham et al. showed that IL-22 is not essential in the immunity against the intracellular pathogen *Listeria monocytogenes* [213]. Moreover, in mice, IL-22 is unnecessary for host defence against intracellular pathogens during intravenous infection with *Mycobacterium avium* [214], intraperitoneal infection with *Salmonella enterica* intratracheal [202] and during intra-tracheal infection with *Francisella tularensis* [215]. Based on this observation and the fact that IL-22 induces production of antimicrobial peptides from epithelial cells, it became clear that the major function of IL-22 is the control of extracellular bacteria.

In the mouse model of *Citrobacter rodentium* infection that specifically infects epithelial cells in the murine colon, which is normally a self-resolving infection, but in IL-22-deficient mice turned deadly within the second week post-infection. In this model, IL-22 contributes to host defence mainly by inducing anti-microbial Reg peptides (RegI, RegII, RegIIIα, RegIIIβ, RegIIIγ, RegIIIδ) production from epithelial cells [162]. In addition, IL-22 is involved in the resistance against pulmonary infection with gram-negative bacterium - *Klebsiella pneumonia*, since neutralization of IL-22 resulted in death of animals within 24 hours post infection. Here, IL-22 together with IL-17 induces the expression of cytokines, chemokines and lipocalin-2 that directly kills bacterium [169]. Finally, beneficial role of IL-22 in control of infection was shown for *Staphylococcus aureus* infection in the skin and lung [216] as well as for *Bacillus subtilis* induced lung inflammation [217].

Role of IL-22 in immunity against fungal infection was also investigated. First of all, in a model of lung infection with *Aspergillus fumigatus*, IL-22 contributes to the protective immunity and control of fungal burden in a Dectin-1/IL-23 dependent manner [218]. Secondly, IL-22 is induced in the stomach of mice infected with *Candida albicans* via an intragastric route, and is indispensable in preventing the dissemination of *C.albicans* to the stomach and kidney [219]. On the contrary, IL-22 has a minor role against *Candida* infection when the yeast is applied via the oropharyngeal route [220] or in the skin model [221]. In humans, role of IL-22 in control of *Candida albicans* infection is not clear. It has been reported that, patients that have high titre of anti-IL-22, anti-IL-17A and anti-IL-17F auto-antibodies are susceptible to develop chronic mucocutaneous candidiasis (CMC) [222].

Similarities between IL-22 and type III interferons (IL-28A, IL-28B, IL-29) in terms of sharing common receptor subunit IL-10R2 and acting on the same cells, suggested that IL-22 may play a role in anti-viral immunity [121]. Indeed, in influenza virus infection mouse model, IL-22 plays a role in protection of tracheal epithelial cells and additionally prevents secondary bacterial infection with *S. aureus* [216, 223]. However, IL-22 does not act directly on the virus, but rather limits tissue damage caused by virus [223]. In human immunodeficiency virus (HIV) infection in humans, in the chronic phase, reduction of Th22 cells is observed and it correlates with loss of epithelial cell integrity. As a result, chronic immune activation is induced by increased microbial dissemination to gut [224].

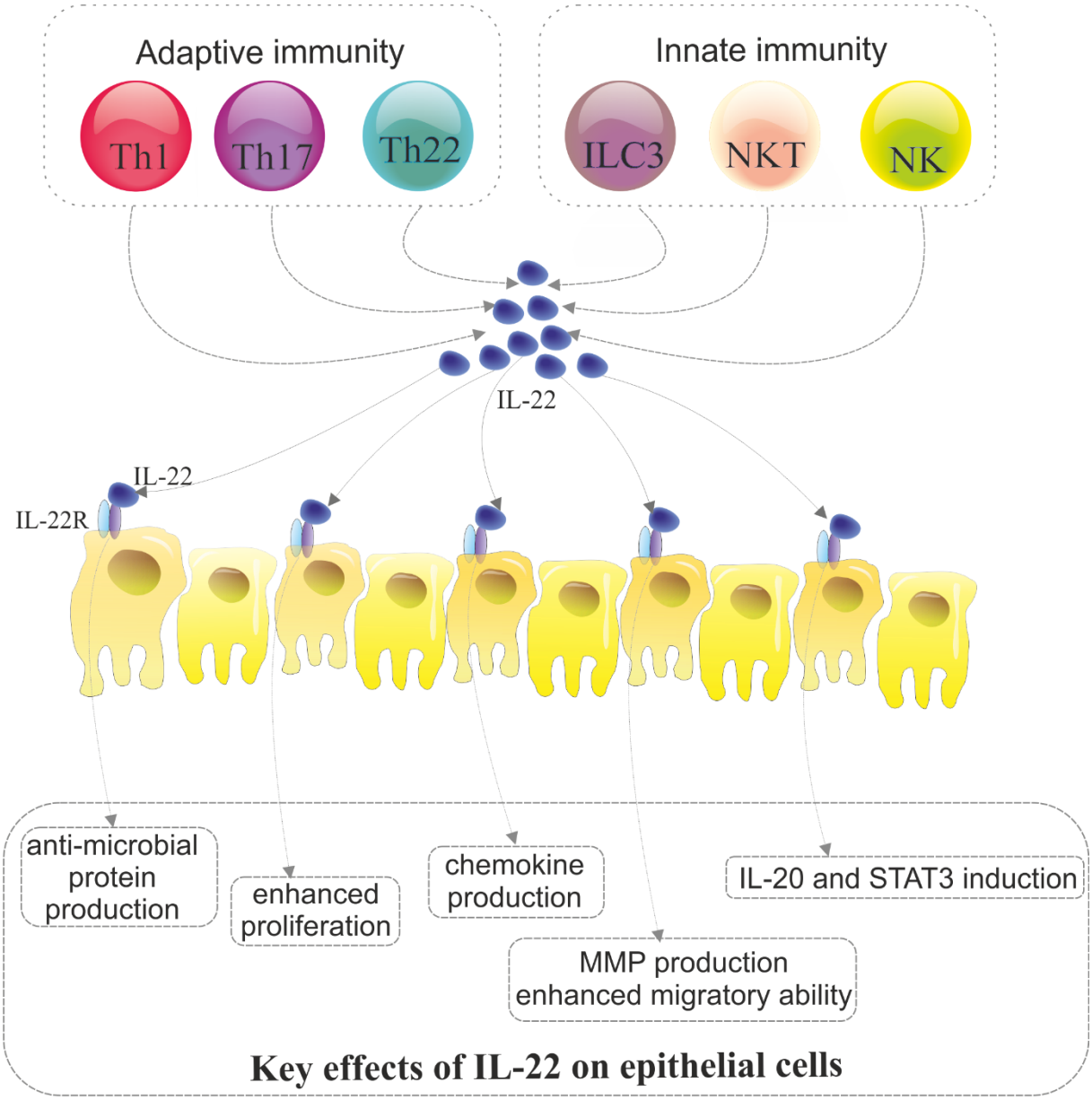


Figure 4. IL-22-producing T cells and effect of IL-22 on epithelial cells.

5.5 Immunology of palatine tonsils.

Palatine tonsils (PTs) are a part of mucosa-associated lymphoid tissue (MALT) in the human pharynx named the Waldeyer`s ring [225]. Besides palatine tonsils Waldeyer`s ring consist of: nasopharyngeal tonsil (adenoid - attached to the roof of the pharynx), paired tubal tonsils (at the openings of the Eustachian tubes) and lingual tonsil (at the back of the tongue) [226]. Considering the fact that PTs are easily accessible, palatine tonsils are the best studied among all tonsils. Palatine tonsils are positioned in the entry of respiratory and gastrointestinal tracks, the place where, just before they are exposed to digestive enzymes and acidic gastric secretion, foreign antigens and substances from food and air come into contact with the mucosal tissues. This is the strategic region where the immune responses against diverse antigens that enter the body through the mouth and nose are initiated [227]. Adenoids as well as palatine tonsils may be removed, however fully functional lingual and tubal tonsils might take over their role [228]. The characteristic feature of palatine tonsils is the formation of deep tubular crypts, that extends the external surface of tonsil up to 300 cm2 [229]. Long-term contact with antigens and direct stimulation of immune cells with food and air-born allergens is possible due to highly cryptic surface. The outer surface of the palatine tonsils is covered by a stratified squamous non-keratinized epithelium. Contrary to this, the epithelium of crypts is called lymphoepithelium, as it is infiltrated with non-epithelial cells, mainly lymphocytes [226]. In the reticulated epithelium, cells, which are functionally matching intestinal M-cells were found [230]. As they possess remarkable potential to transcytose a broad range of particles and soluble material without their degradation, they translocate antigens to subepithelial lymphoid tissue [231]. Immune cells, mainly lymphocytes are found in all the compartments of the tonsils including the lymphoepithelium (the intraepithelial leukocytes - IELs), the inter-follicular regions and the follicles. Among all IELs, 50% are B cells and smaller number of T cells, mostly CD4+ rather than CD8+

T cells. Similarly, the secondary lymphoid follicles are mainly populated by B cells; intense maturation and differentiation takes place there. Beside B cells in the germinal center of follicular T cells and follicular DC`s are found [226, 232]. Interestingly, recently McClory et al. identified 5 tonsillar T cell developmental intermediates and proved that each of them resembles its thymic counterpart. With this work it has been proven that full stepwise program of T cells development may take place in human tonsils [233]. Moreover, tonsils have been shown as the potential first-line organ involved in tolerance via generation of allergen specific FOXP3+ T regulatory cells [228]. As described above, from immunological point of view palatine tonsils are very interesting organs but yet not fully thoroughly investigated.

5.6 microRNAs - biogenesis and function

Human genome studies have revealed that only a small fraction of transcribed genes is further translated and all non-coding transcripts are named as ncRNA (non-coding RNA) [234, 235]. ncRNA has been organized into the groups based on their position in genome, function or length of the transcript. The division based on the length of the transcript divides ncRNA into: small and long ncRNA. The group of small ncRNA consists of: 1) microRNAs (18–25 nt), 2) small-interfering RNAs (19–23 nt), 3) piwi-interacting RNAs (26–30 nt), 4) small nuclear and nucleolar RNAs (60–300 nt), 5) promoter-associated small RNAs (20–200 nt), 6) transcription initiation RNAs (18 nt), 7) centromere repeat-associated small interacting RNAs (34–42 nt), 8) telomere-specific small RNAs (24 nt), and 9) pyknons (variable in size) [236].

Discovered for the first time in *Caenorhabditis elegans* [237] almost 20 years ago, microRNAs are nowadays recognized as posttranscriptional gene expression regulators. Genes coding microRNAs are mostly non-coding genes from, which the only product is the miRNA. Additionally, miRNA genes may be located within un-translated regions of a protein coding genes or gene introns [238]. MicroRNA genes are transcribed by RNA polymerase II into primary the miRNAs transcript (pri-miRNAs) [239]. As it happens for the protein coding mRNA, the pri-miRNA is spliced, capped and polyadenylated [240]. The primary miRNA transcript is converted into active miRNA in two endonuclease dependent steps. During the first step, pri-miRNAs are bound to mRNA binding protein - DGCR8, which is associated with the Drosha enzyme [241]. Ribonuclease III (RNase III) activity of Drosha enzyme cleaves flanking sequences of pri-miRNA and releases hairpin structures known as precursor miRNA (pre-miRNAs) [242]. Next, pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5 [243]. The second step, which takes place in the cytoplasm, RNase III Dicer cleaves off the hairpin loop of the pre-miRNA, and releases the mature miRNA [244]. Formed in this way duplex miRNA separates

and one of single stranded miRNA join with Argonaute (AGO) proteins from miRISC, the miRNA-induced silencing complex. The other strand, which does not form miRISC, is typically degraded. Nevertheless, in case of some miRNAs, both strands of mature miRNA can be loaded into miRISC. The strand from 3` end of hairpin is named "3p" and from 5` end is named "5p" [245]. The core of miRISC, formed by mature miRNA bound to AGO proteins, seeks for target mRNA to which mature miRNA is specific. The major determinants of target mRNA recognition are located in the "seed region" in the position 2-8 from the 5` end of miRNA. However, the 3` end of miRNA can contribute to target recognition as well [246]. Depending on the location of complementarity, the mRNA target may be cleaved directly by the endonuclease activity of AGO – complementarity in central region, or other protein complexes, like GW182, CCR4-NOT, may be recruited to miRISC to remove poly (A) tail, what leads to translational repression and increased mRNA degradation [247, 248].

5.6.1 MicroRNA in T cells function and differentiation

Specific patterns of miRNA expression in murine haematopoietic system, which have been identified in systemic mRNA profiling studies, proposed that miRNAs may have a role in development and effector function of cell lineages [249]. Since then, extensive profiling of naïve, activated and differentiated human CD4+T cells took place [250]. As predicted, decreased survival and proliferation of T cells upon activation was observed in T cells where Dicer or Drosha enzymes as well as DGCR8 subunits were deleted [251, 252]. However, increase cytokine production and differentiation into T helper cells was observed in miRNA deficient CD4+ T cells, suggesting that miRNA may be important in maintenance of T cells in the naïve state [252]. Similarly, with a few exceptions namely miR-155 and group of miR-17-92 were induced, most of miRNAs that are expressed in resting T cells are downregulated after T cells activation [253]. Additionally, upon activation CD4+ T cells shorten mRNA 3`UTRs and specific

miRNA binding sites are lost, that as a result protects target mRNA from miRNA-mediated degradation [254]. MiR-29ab, miR-146a, miR-155 and are the best studied miRNAs in T cells [255].

The role of miR-17-92 gene cluster was studied in detailed in PI3K signalling during T cell activation and signal transduction. In mouse models where miR-17-92 cluster was overexpressed during thymocyte development, CD4+ T cells expanded and mice developed systemic autoimmunity. The main effect was observed because miR-17-92 promotes CD4 T cells proliferation in response to TCR stimulation and inhibits activation induced cell death without the need for co-stimulation signals provided by CD28-CD80/CD86 interactions [256, 257].

The other known regulator of T cell signalling that has an effect on T cells development is miR-181. Inhibition of miRNA-181 in thymocytes impaired T cells selection and decreased TCR sensitivity. Oppositely, overexpression of miR-181 in mature T cells reduces TCR threshold for activation and increases sensitivity to presented antigens in the periphery [258].

During antigen recognition and T cells activation, IL-2 produced by T cells function in the autocrine manner to promote cells proliferation. Regulation of downstream signalling of IL-2R by miRNA-182 has been described. miR-182 enhances clonal selection by inhibiting FOXO1, the T cell proliferation limiting transcription factor that is highly expressed in naïve T cells [259]. On the contrary miRNA-146a negatively regulates IL-2 production in T helper cells [260]. Reduction of IL-2 production and inhibition of cell activation occurs as well when miR-181d directly targets IL-2 mRNA in T helper cells [261]. miR-184 and miR-31 instead of directly targeting IL-2 mRNA, regulate the main IL-2 transcription factor – NFAT and inhibits – miR-184 or activates – miR-31 IL-2 production [262, 263]. The biogenesis of miRNA is shown on Figure 5.

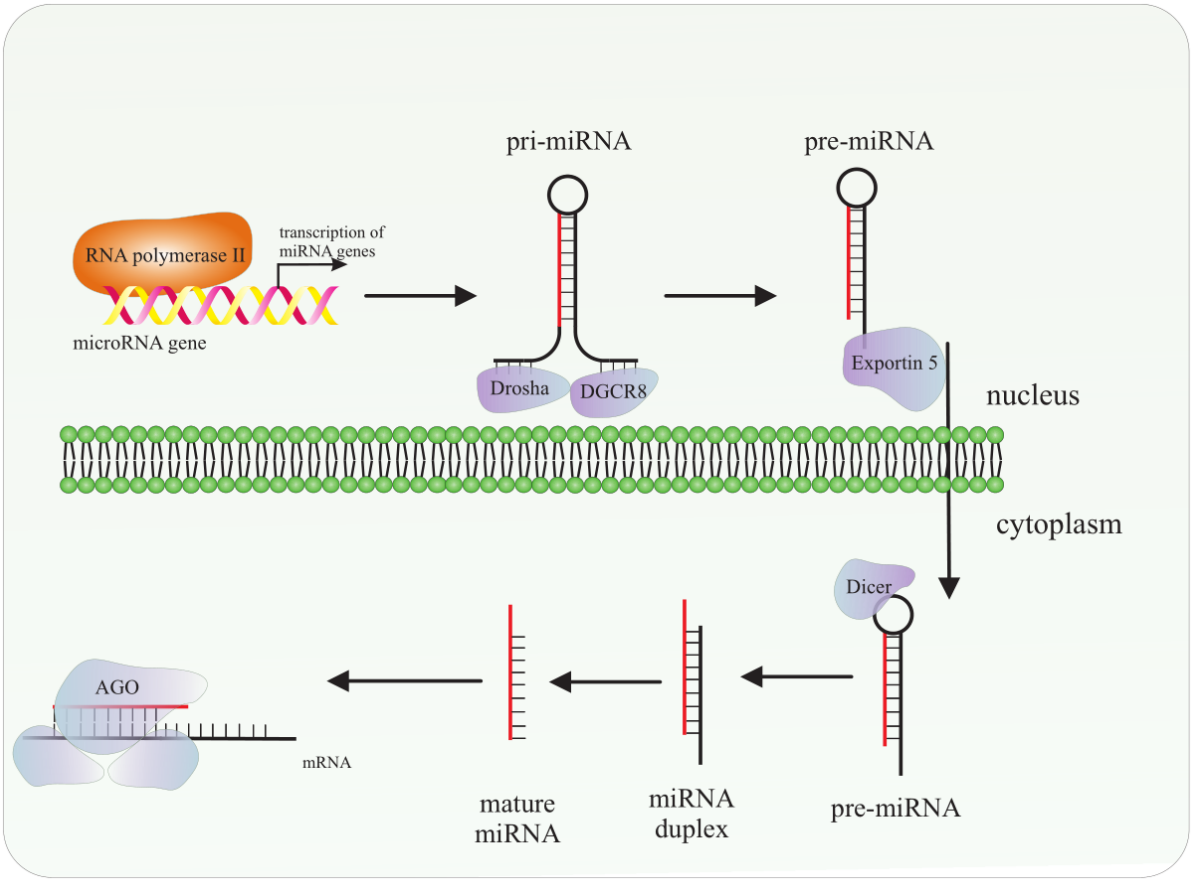


Figure 5. Biogenesis of microRNA (adapted from [264])

5.6.2 Role of miRNAs in Th1, Th2 and Th17 cell differentiation

The most potent, direct inhibitors of Th1 differentiation and IFN-γ production are miR-29a and miR-29b [265]. T-bet, the key transcriptional factor of Th1 cells and Eomes, the regulator of IFN-γ production, both are targeted by miR-29a/b, that results in the inhibition of IFN-γ production [266]. Interestingly, miR-29 can directly target mRNA for IFN-γ [266, 267] as shown in *in vivo* infection with *Listeria monocytogenes* and *Mycobacterium bovis* models. In the models mentioned above, IFN-γ production and miR-29 expression negatively correlated in natural killer as well as T cells [266]. Additionally mice expressing a "miRNA sponge", a transgene with multiple miR-29 binding sites that reduced

miR-29a levels, controlled *Listeria monocytogenes* infection due to increased IFN-γ production from T cells [267]. Nevertheless, miR-29a was induced by IFN-γ, in the process of negative feedback loop that may play a role in autoimmune diseases [267].

Other various miRNAs may indirectly influence Th1 cells differentiation. CD4+ T cells deficient in miR-146a produce more IFN-γ as compared with non-manipulated cells. The main effect of miR-146a is the regulation of STAT1, Traf6 and Irak1 [268, 269]. On the contrary, miR-155 enhances Th1 mediated immunity, mainly by inhibiting SOCS1 (Suppressor of cytokine signalling 1) and SHIP-1 molecules involved in suppression of cytokine signalling [270, 271]. It has been shown that in mice deficient in miR-155, polarization and differentiation of T helper cells is directed into Th2 cells with high levels of IL-4 and IL-5 [272, 273]. miR-155 targets directly the activator of IL-4 promoter, however the promotion of Th1 cells by miR-155 takes place only in the absence of Th2 polarizing cytokines [273]. miR-17-92 cluster is another activator of human Th1 differentiation. Under Th1 polarizing conditions, naïve transgenic CD4+T cells produced large amounts of IFN-γ [257] and the deletion of miR-17-92 resulted in reduction of IFN-γ secreting Th cells [256].

The indirect effect of microRNA`s on differentiation of Th1 cells is delivered by miR-21, which modulates IL-12 production by dendritic cells and thereafter regulates Th1 responses. miR-21 directly targets mRNA for the IL-12p35 subunit. Increased IL-12 production by DC`s is observed *in vivo* with miR-21 deficiency, that results in enhanced Th1 development and delayed-type hypersensitivity responses [274].

As compared to regulation of Th1 differentiation by miRNAs, regulation of Th2 differentiation is less well understood. Th2 polarization of mouse CD4+ T cells is promoted by miR-126 via direct targeting of POU2F3 – the activator of the PU.1 transcription factor [275]. Moreover, inhibition of miR-126 reduced Th2 responses to dust mite antigens *in vivo* [275]. In the same model, increased

expression of miR-126 in the airway wall was reported. Interestingly, administration of the *antagomir,* which directly targets miR-126 decreased AHR (airway hyper-responsiveness) and recruitment of eosinophils to lungs [275]. In the microRNA overexpression experiments it has been shown that Th2 cells differentiation may be affected. Overexpression of miR-21 increased Th2 differentiation, however overexpression of miR-27 and miR-128 decreased secretion of Th2-related cytokines by activated T helper cells [276, 277].

Polarization of naive T cells into Th17 cells is enhanced mainly by miR-155, miR-301 and miR-326. In the murine model of experimental autoimmune encephalomyelitis (EAE) and in patients with multiple sclerosis, expression of miR-326 was up-regulated in CD4+ T cells and correlated positively with disease severity and IL-17A mRNA expression [278]. Furthermore, direct *in vivo* targeting of miR-326 with "sponge miRNA" reduced severity of EAE in mice. Similarly, *in vitro* blocking of miR-326 reduced Th17 cell differentiation. Mechanistically, Ets1 – the main negative regulator of Th17 cells differentiation is directly targeted by miR-326, which leads to increase Th17 cells differentiation [278].

It has been reported that CD4+ T cells from mice with EAE expressed enhanced levels of miR-155. As expected, miR-155-deficient mice showed less inflammation, fewer Th17 cells in the brain and other tissues, and were resistant to EAE [279, 280]. Beyond contributing to Th17 mediated autoimmune diseases in the T cell dependent manner, miR-155 stimulates production of Th17 polarizing cytokines, IL-6, IL-21, and IL-23, by activated dendritic cells [280]. Additionally, defective Th17 cell responses in mice lacking miR-155 made them resistant to collagen induced arthritis as well as chronic gastritis and colitis in response to *Helicobacter pylori* [281, 282]. In summary, as described in previous paragraphs, miR-155 may play role in both Th1 and Th17 cells differentiation.

Finally, the last known miRNA that promotes Th17 cells differentiation is miR-301a. Through targeting Pias3, inhibitor of STAT3 transcription factor critical for IL-6 and IL-23 induced Th17 cells development, miR-301a drives naive T cells differentiation into Th17 cells [283].

6 Results

6.1 Increased microRNA-323-3p in IL-22/IL-17-producing T cells and asthma: a role in the regulation of the TGF-β pathway and IL-22 production

J. Kärner,[1,*] M. Wawrzyniak,[2,*] S. Tankov,[1] T. Runnel,[1,3] A. Aints,[4] K. Kisand,[1] A. Altraja,[5,6] K. Kingo,[7,8] C. A. Akdis,[2] M. Akdis[2] & A. Rebane[1]

[1]Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia.
[2]Swiss Institute of Allergy and Asthma Research (SIAF), University of Zürich, Davos, Switzerland.
[3]Institute of Molecular and Cellular Biology, University of Tartu, Tartu, Estonia.
[4]Department of Obstetrics and Gynecology, Medical Faculty, University of Tartu and Competence Centre on Health Technologies, Tartu, Estonia.
[5]Department of Pulmonary Medicine, University of Tartu, Tartu, Estonia.
[6]Lung Clinic, Tartu University Hospital, Tartu, Estonia.
[7]Department of Dermatology and Venereology, University of Tartu, Tartu, Estonia.
[8]Dermatology Clinic, Tartu University Hospital, Tartu, Estonia.

*These authors contributed equally to this work

Correspondence: Ana Rebane, Institute of Biomedicine and Translational Medicine, University of Tartu, Ravila 14B, 50414 Tartu, Estonia; Tel: +372 7 374 419; Fax: +372 7 374 207; E-mail: ana.rebane@ut.ee

Running title: microRNA-323-3p in IL-22/IL-17-producing T cells

Key words: Th17 cell, Th22 cell, allergy, non-coding RNA, SMAD

Abbreviations

AD, atopic dermatitis; CDKN1B, cyclin-dependent kinase inhibitor 1B; IL, Interleukin; miRNA, microRNA; SMAD, mothers against decapentaplegic homologue; PBMC, peripheral blood mononuclear cell; Th, T helper; TGF-β, Transforming growth factor-β; 3'UTR, 3' untranslated region

ABSTRACT

Background:

IL-22- and IL-17-producing T cells have important roles in allergic diseases. MicroRNAs (miRNAs) are post-transcriptional gene expression regulators, which modulate numerous biological processes, including immune responses in allergic diseases. There is little known about the functions of miRNAs in IL-22/IL-17-producing T cells.

Material and Methods:

IL-22- and IL-17-positive T cells were sorted from human peripheral blood mononuclear cells (PBMCs) by means of intracellular staining. miRNA expression profiles were detected with TaqMan array microfluidic cards. For functional studies, T cells and human embryonic kidney 293 cells were transfected with miRNA mimics. The expression of miRNAs and protein coding genes was analyzed using RT-qPCR and/or enzyme-linked immunosorbent assay in T cell subsets, T cell culture supernatants and PBMCs from patients with asthma and atopic dermatitis.

Results:

The increased expression of miR-323-3p and non-coding RNA nc886 and the reduced expression of miR-146a, miR-93, miR-181a, miR-26a and miR-874 were detected in IL-22-producing T cells. The pathway analysis of the putative targets suggested that these differentially expressed miRNAs could impact the proliferation, differentiation and effector functions of T cells. Further analyses showed enhanced expression of miR-323-3p in both IL-22- and IL-17-positive T cells and its capacity to suppress IL-22 production in T cells. Multiple genes from the transforming growth factor β pathway were identified as direct targets of miR-323-3p. An increased expression of miR-323-3p was observed in PBMCs from asthma patients.

Conclusions:

Our data suggest that miR-323-3p may play a role in the regulation of IL-22 production and T cell responses in patients with asthma.

INTRODUCTION

T helper (Th) 17 cells are characterized by the expression of interleukin (IL)-17A (referred to as IL-17 in this study) and IL-17F [284, 285]. Depending on the environmental effectors, Th17 cells can also produce different amounts of IL-22, IFN-γ, IL-10 as well as other cytokines. For example, in the presence of transforming growth factor (TGF)-β, Th17 cells are known to produce more IL-10 and in the absence of TGF-β more IL-22 [286]. More recently, a distinct T cell-subset producing IL-22, but not IL-17 or IFN-γ, was defined [287, 288] and was found to play roles in the immune responses of the skin including the pathogenesis of atopic dermatitis (AD) [147, 177]. IL-22 has been shown to suppress antigen-induced airway inflammation in mice while being linked to tissue protection and regeneration [284, 285, 289]. IL-17 has been demonstrated to be central in neutrophilic airway inflammation [290, 291].

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression that mainly cause gene silencing by the degradation of target mRNAs and the inhibition of translation [292]. The function of numerous individual miRNAs has been demonstrated in the regulation of differentiation, phenotypic stability and plasticity of T cells [264]. For instance, the differentiation of Th17 has been shown to be dependent on the transcription factor retinoic acid receptor-related orphan receptor γt (RORγt) [92], as well as on several miRNAs, such as miR-155 [280], miR-10a [293] and miR-326 [278]. Recent studies demonstrate that miRNAs also play important roles in the regulation of immune responses in allergic diseases [294-296], which partially might be due to the impact on differentiation, phenotypic stability and plasticity of T cells [264]. The role of miRNAs in the development and regulation of IL-22-producing T cells have not been described previously.

In the present study, we analyzed the expression of miRNAs in IL-22-positive T cells and peripheral blood mononuclear cells (PBMCs) from patients with asthma and AD. We demonstrate increased expression of miR-323-3p in

IL-22/IL-17-producing T cells, its capacity to suppress multiple factors from the TGF-β pathway and IL-22 production in T-cells from heathy donors. In addition, we report increased expression of miR-323-3p in the peripheral blood mononuclear cells (PBMCs) from patients with asthma.

MATERIALS AND METHODS

Study participants

This study was approved by the Ethical Review Committee on Human Research of the University of Tartu. All participants provided a signed informed consent form. None of the patients or control individuals was under the treatment with anti-histamines or systemic corticosteroids either currently or within three weeks prior to the study. Patients with pulmonary physician-diagnosed asthma according to the Global Initiative for Asthma (www.ginasthma.org) aged below 55 years were included. Eight out of 10 patients with asthma had known allergies, 9 out of 10 used inhaled corticosteroids on a regular basis. Seven out of 10 patients included with dermatologist-diagnosed AD (age range 21-58) had the confirmed diagnosis of the allergic type of AD based on an increased level of IgE. All patients with AD had an exacerbation with 1-2-week duration prior to their recruitment into the study. The control individuals were randomly selected from the general population of the same age and were free of allergic diseases.

Isolation of PBMCs

Human PBMCs were isolated from the peripheral blood of healthy adult volunteers or patients with asthma or AD by using density gradient centrifugation on Ficoll (Biochrom, Berlin, Germany), as described previously [297, 298].

Intracellular staining and cell sorting

Human PBMCs were cultured in supplemented RPMI-1640 (cRPMI) medium for 3 days as described previously [297] in the presence of 20 μg/ml TLR3 ligand Poly I:C (Sigma-Aldrich), IL-7 (40 ng/ml), and IL-23 (10 ng/ml). On day 3, the cells were stimulated with PMA (25 ng/ml) and Ionomycin (1 μg/ml) in the presence of Brefeldin A (2 μg/ml) for 6 h, followed by staining

with fixable viability dye eFluor 780 (eBioscience, Aachen, Germany) and PerCP-Cy5,5-conjugated anti-CD3 (Biolegend San Diego, CA, USA). Then, the cells were permeabilized using cytofix/cytoperm kit (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer's instructions, stained intracellularly with mouse anti-human PE-conjugated anti-IL-22 antibody (eBioscience) and with Alexa Fluor 488-labeled mouse anti-human IL-17A Ab (Biolegend), and sorted by a BD FACSAria™ II.

miRNA profiling

For miRNA profiling, total RNA from 10^5-2×10^6 cell for each sample was extracted using a miRNAeasy Mini Kit (Qiagen, Valencia, CA, USA) in the presence of 0.8 µg of MS2 carrier RNA (Roche, Basel, Switzerland). Reverse transcription (RT) with Megaplex™ Primer Pools (Human, A v2.1), pre-amplification of RT products with Megaplex™ PreAmp Primers (Human Pool A, v2.1) and miRNA profiling with TaqMan® Array Human MicroRNA. A microfluidic Cards (v2.0) were performed using the 7900HT Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA) and analyzed according to manufacturers' instructions. A total of 68 miRNAs detected with a cycle value of less than 30 were subjected to further analysis. The data were normalized against miR-19b. Arbitrary units were calculated according to the following formula: arbitrary units = $2^{-\Delta Ct} \times 10.000$.

miRNA target selection and pathway analysis

miRNA targets containing miRNA binding sites with a total context score of less than -0.2 and the targets conserved among vertebrates were selected using Targetscan 6.2 [299]. Out of the selected targets, only the genes expressed in unstimulated CD4+ cells (9,539 genes with detection *P*-value of $P<0.05$ according to A-MEXP-1173) were subjected to the pathway analysis. Pathway analysis was performed with g:Profiler (http://biit.cs.ut.ee/gprofiler), which retrieves the most significant Gene Ontology (GO) groups and the Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathways and enables one to estimate significance of the search results by calculating the enrichment *P*-value with the one-tailed Fisher's exact test [300].

Luciferase assay

300-500 nt long 3' untranslated region fragments of SMAD2, SMAD3, SMAD5 and CDKN1B were PCR-amplified using human genomic DNA as a template and cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) in NheI and SalI sites. The following primers were used: SMAD2 For 5'-ATT GCT AGC GTG GAA TCT GTT TCC TAT AT-3', SMAD2 Rev: 5'-ATT GTC GAC CTA CAG GAA AAT CTG CTT CT-3', SMAD3 For: 5'-ATT GCT AGC GTC TTC ACA ATG TAT TTT CAT CA-3', SMAD3 Rev: 5'-ATT GTC GAC CAG AGG ACC CTT GTG GA-3', SMAD5 For: 5'- ATT GCT AGC GCA TAT ACA GTG AAG AGT AA-3', SMAD5 Rev: 5'-ATT GTC GAC GCA TCT AAG TCC AAG TCC AAG TCA C-3', CDKN1B For: 5'-ATT GCT AGC GAT AAG TGA AAT GGA TAC TAC AT-3', CDKN1B Rev: 5'-ATT GTC GAC CTT TAT TGA TTA CTT AAT GTG TAA c-3', CDKN1B II For: 5'-ATT GCT AGC GAT GTA GCA TTA TGC AAT TAG G-3' and CDKN1B II Rev 5'-ATT GTC GAC CAG CTA TCT AAC AAA CTT TAG AT-3'. The transfections were carried out in 24-well plates using 0.8 µl of siPORT NeoFX (Life technologies, Grand Island, NY, USA), 30 nM of mirVana® miRNA mimics hsa-miR-323a-3p and a Negative Control #1 (Life Technologies), 50 ng of the reporter plasmid, and $2x10^4$ human embryonic kidney epithelial 293 cells in 0.6 ml medium for 24 hours. Firefly and renilla luciferase activities were measured using Promega dual luciferase assay and firefly luciferase activities were normalized to the values of the renilla luciferase.

T cell isolation and transfection with miRNA mimics

T cells were isolated from frozen healthy blood donors' PBMCs using Dynabeads Human T-Expander CD3/CD28 (Life Technologies, Carlsbad, CA, USA) beads, according to the manufacturer's protocol. Briefly, PBMCs were thawed with adding warm TexMACS Medium (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), suspended, centrifuged and washed with 1% of bovine serum albumin (BSA) in phosphate buffered saline (PBS). After that, the cells were counted, mixed with Dynabeads in a 1:1 ratio in the total volume of 1 ml 1% BSA in PBS and rotated at 1 rpm for 30 minutes at 4°C. The bead-bound cells were collected in the magnetic field, resuspended in TexMACS medium, counted and incubated at 37°C for 24 hours prior the transfection. The transfection was performed with Neon electroporation transfection system (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol with minor modifications. Transfections were performed using 2-4 x 10^7 cells/ml and miRNA concentration of 500 nM in 10 µl "R buffer" (Life Technologies) and the Neon transfection system setting 1,550V with three 10-ms pulses. Pre-miR™ Precursors Molecule hsa-miR-146a and mirVana miRNA Mimics hsa-miR-323a-3p and the Negative Control #1 (all from Life Technologies) were used for the transfection. The transfected T cells were cultured at the starting concentration of 10^6 cells/ml in TexMACS Medium (Miltenyi Biotec) for 72 hours at 37°C with an additional stimulation of PMA (25 ng/ml) and Ionomycin (1 ug/ml) during the last 6 hours.

Isolation of total RNA, cDNA synthesis and reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted using miRNAeasy Mini Kit according to the manufacturers' instructions (Qiagen Valencia, CA, USA). For mRNA expression analysis, cDNA was synthesized from 100-900 ng of total RNA with oligo-dT, RiboLock RNase Inhibitor, RevertAid First Strand cDNA Synthesis Kit and dNTP Mix according to the manufacturer's instruction (Thermo

Scientific, Waltham, MA, USA). RNA concentration and quality were assessed with the NanoDrop ND-1000. 5xHOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne, Tartu, Estonia) and ABI Prism 7900 or Via7 were used for qPCR. The relative gene expression levels were normalized to the level of human EEF1A1 and calculated using the comparative Ct (ΔΔCt) method (Life Technologies, Carlsbad, CA, USA). SMAD2 for: GAA AGG GTG GGG AGC AGA AT, SMAD2 rev: TCC AAC CAC TGT AGA GGT CCA, SMAD3 for: CCT AGG GCT GCT CTC CAA TG, SMAD3 rev GAC CTC CCC TCC GAT GTA GT, SMAD5 for: GTT TGC TCA GCT TCT GGC TC, SMAD5 rev: CGG TGA TAT TCT GCT CCC CAA, CDKN1B for: TCC GGC TAA CTC TGA GGA CA and CDKN1B rev: GGT TGC AGG TCG CTT CCT TA were designed with the assistance of Primer 3 and were ordered from TAG Copenhagen (Copenhagen, Denmark). qPCR primers for human CCL5, IRAK1 and EEF1A are described before [301]. miRNA qPCR was carried out using TaqMan microRNA Assays (Life Technologies) and using 5× HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis BioDyne). miRNA RT-qPCR results were normalized to miR-19b. Relative expression is shown compared to the mean of control experiments or the control group (=1) both in mRNA and miRNA RT-qPCR.

Enzyme-linked immunosorbent assay (ELISA)

IL-17A and IL-22 amounts in the supernatants of T cells were measured using READY- SET- GO! Human Interleukin-17A (eBioscience, Aachen, Germany) and Human IL-22 DuoSet (R&D Systems, Minneapolis, MN, USA) ELISA kits according to manufacturers' protocols. Briefly, 96-well half-area microplate wells (Greiner Bio-One) were covered with 30 µl of purified anti-IL-17A (dilution 1:250 in coating buffer) or anti-IL-22 (2 µg/ml in PBS) antibodies and incubated overnight at 4°C (IL-17A) or at the room temperature (IL-22). Then, the plates were washed, blocked with 100 µl of Assay Diluent

(IL-17A) or 2% BSA (IL22) for 1 hour, washed again, incubated with 30 µl of T cell culture supernatants overnight at +4°C and further manipulated as described in manufacturers' protocols.

Statistics

Statistical analysis was performed using unpaired Students *t*-test. The results were considered significant at $P<0.05$.

RESULTS

Differential expression of miRNAs in IL-22-positive T cells

To find miRNAs characteristic of IL-22 production in T cells, IL-22-positive ($CD3^+IL\text{-}22^+$) and IL-22-negative ($CD3^+IL\text{-}22^-$) T cell populations were sorted from PBMCs of healthy donors using an intracellular staining approach (Fig. S1), and miRNA expression profile of both cell populations was determined using human TaqMan MicroRNA microfluidic cards. A total of 18 miRNAs were found to be differentially expressed with more than a 2-fold difference in the $CD3^+IL\text{-}22^+$ compared to $CD3^+IL\text{-}22^-$ T cells (Fig. 1A). In this set of miRNAs, miR-323-3p, miR-886-5p, and miR-886-3p were significantly up-regulated and miR-874, miR-26a, miR-181a, miR-93, and miR-146a were down-regulated in $CD3^+IL\text{-}22^+$ cells (Fig. 1B). Additional analysis of IL-22-single positive, IL-17-single positive, IL-17- and IL-22-double positive, and IL-17- and IL-22-double negative T cell populations ($CD3^+IL\text{-}17^-IL\text{-}22^+$, $CD3^+IL\text{-}17+IL\text{-}22^-$, $CD3^+IL\text{-}17^+IL\text{-}22+$ and $CD3^+IL\text{-}17^-IL\text{-}22^-$) (Fig. S1B) confirmed that in IL-17- and IL-22-producing T cells, the expression of miR-323-3p and miR-886-5p was increased in comparison to the non-producing cells (Fig. 1C).

miRNAs differentially expressed in IL-22-positive T cells may influence T cell functions

To analyze whether miRNAs differentially expressed in IL-22-positive T cells could potentially influence the differentiation and functions of T cells, we performed pathway analysis with g:Profiler for the best scored and conserved miRNA targets defined by Targetscan 6.2 and expressed in the CD4(+) T cells. The pathway analysis revealed a significant overlap between miR-323-p, miR-26a, miR-874, miR-93 and 146a targets with numerous GO groups and KEGG pathways that impact T cell development, immune responses to pathogens and cytokines, tissue regeneration and apoptosis (Table 1). miR-886-5p and miR-886-3p were excluded from the pathway analysis, as they were

recently shown to represent a 139 nt long noncoding RNA nc886 that functions as a repressor of interferon-induced, double-stranded RNA-activated protein kinase [302].

miR-323-3p targets genes from TGF-β signaling pathway and inhibits the production of IL-22 in T cells

The integrated pathway and target analysis suggested that miR-323-3p could modulate the expression of the genes regulating cellular responses to TGF-β (Table 1), a cytokine that plays a role in Th17 cell differentiation and in the regulation of IL-22 production by Th17 cells [284]. We therefore analyzed the capacity of miR-323-3p to influence putative direct targets from the TGF-β signaling pathway. In particular, we cloned fragments of 3' untranslated regions (3' UTRs) of SMAD 2 (mothers against decapentaplegic homologue 2), SMAD3, SMAD5 and cyclin-dependent kinase inhibitor 1B (CDKN1B, also known as p27) containing predicted miR-323-3p binding sites (Table 2) into the luciferase reporter vector. The cloned reporter plasmids were transfected together with the miR-323-3p or the control mimic into human embryonic kidney 293 (HEK293) cells followed by the luciferase assay. miR-323-3p suppressed the luciferase activity of the reporters containing 3' UTR regions of SMAD3, SMAD5 and a conserved binding site of CDKN1B (Fig. 2A). To clarify whether miR-323-3p has capacity to influence the expression of these direct targets in T cells, we next transfected bead-purified CD3 positive T cells together with miR-323-3p mimic, negative control mimic or precursor for miR-146a, which we also observed to be differentially expressed in IL-22-positive T cells (Fig. 1B). As demonstrated in Fig. 2B, miR-323-3p suppressed SMAD2, SMAD3 and CDKN1B and moderately influenced SMAD5 mRNA expression in cultured CD3(+) T cells. miR-146a, which was used as a positive control inhibited previously described direct targets IL-1 receptor–associated kinase 1 (IRAK1) and CCL5 [301, 303]. Remarkably, the transfection of

miR-323-3p mimic suppressed the expression of IL-22 mRNA (Fig. 2D) and the secretion of IL-22 protein (Fig. 2E), while the IL-17A levels were not significantly changed. Transfection of miR-146a precursor did not significantly influence IL-17A and IL-22 mRNA and protein levels (Fig. 2D-E). These results together demonstrate that miR-323-3p targets multiple factors from the TGF-β pathway and strongly inhibits IL-22 production in T cells.

The expression of miR-323-3p is increased in PBMCs from patients with asthma

To study whether miR-323-p expression is altered and can potentially impact immune responses in allergic diseases, we next analyzed the expression of miR-323-3p in PBMCs from patients with asthma and AD and from healthy controls. We observed significantly increased expression of miR-323-3p in PBMCs from patients with asthma compared to control individuals (Fig. 3). The level of miR-323-3p in PBMCs from AD patients was comparable with the control group and did not differ significantly from that of patients with asthma.

DISCUSSION

The functions of miRNAs in the regulation of immune responses in allergic diseases are largely unexplored. In this study, we demonstrated for the first time that the IL-22-producing T cells have a distinct miRNA expression pattern. We show the increased expression of miR-323-3p in IL-22/IL-17-producing T cells and in the PBMCs from patients with asthma. In addition, we demonstrate that miR-323-3p targets multiple genes from the TGF-β signaling pathway and inhibits IL-22 production by T cells. These results together suggest that miR-323-3p might impact T cell responses in asthma.

Previously, several miRNAs have been shown to influence the development, phenotypic stability and functions of T cells. For example, miR-10a limits Th17 cell differentiation and keeps phenotypic stability of inducible Treg cells via targeting the transcriptional repressor Bcl-6 in mice [293]. As another example, miR- 326 targets Ets1, which is a negative regulator of Th17 cell differentiation, leading to increased Th17 cell differentiation *in vitro* [278]. A recent study on miR-155-/- and miR-146a-/- T cells identified that T cell-intrinsic miR-155 is required for type-2 immunity, whereas T-cell-intrinsic miR-146a is needed to prevent Th1/Th17 skewing [304]. Similarly, the pathway analysis we performed suggested that miRNAs differentially expressed in IL-22-positive cells might impact the development, proliferation and immune responses of T cells. Two microRNAs, miR-886-5p and miR-886-3p were excluded from the pathway analysis, as the corresponding PCR probes were recently shown to recognize a 139 nt long noncoding RNA nc886 that functions as a repressor of interferon-induced, double-stranded RNA-activated protein kinase [302]. However, this finding is interesting as the increased expression of nc886 in IL-22-positive cell subset might refer to the reduced interferon response in IL-22-secreting T cells, compared to the rest of the T cell-subsets.

Next, we focused our functional experiments on miR-323-3p as pathway analysis suggested that it may target genes involved in the regulation of the TGF-β pathway. We determined SMAD3, SMAD5 and CDKN1B as the direct miR-323-3p targets, of which SMAD3 and CDKN1B were also inhibited by the transfection of miR-323-3p mimics into T cells. SMAD3 and SMAD5 are regulatory SMADs that upon activation by phosphorylation form homo-oligomeric and hetero-oligomeric complexes with the co-mediator SMAD and translocate to the nucleus, where they function together with transcriptional activators and repressors in the modulation of gene expression [305] (Fig. S2). CDKN1B (also known as p27) controls the cell cycle progression at G1 [306]. This together suggests that miR-323-3p might impact the progression of the cell cycle and the expression of cytokines. Indeed, we observed a strong downregulation of IL-22 mRNA and protein levels when we transfected miR-323-3p into T cells. TGF-β is an important cytokine needed for Th17 cell differentiation, where it has been shown to suppress the production of IL-22 (1). Thus, although miR-323-3p targets several genes from the TGF-β pathway, it cooperates with TGF-β in the suppression of IL-22 production. In addition to miR-323-3p direct targets we characterized in this study, there also exist most likely additional miR-323-3p targets that influence the production of IL-22.

IL-22-producing cells consist of a mixture of IL-22-single positive and IL-22/IL-17 co-producing cells. We observed slightly higher expression of miR-323-3p in IL-17-positive and IL-17/IL-22 double-positive cells, which may suggest that higher miR-323-3p level inhibits the production of IL-22, and thus shift them towards Th17 cells.

miR-323-3p is encoded by 14q32 cluster of miRNAs in humans containing 46 putative miRNA genes that are imprinted and expressed on the maternally inherited chromosome [307]. miRNAs from the 14q32 cluster are deregulated in different cancers including acute promyelocytic leukemia [308] and

aggressive variants of adenocarcinoma of the lung [309]. An increased expression of miR-323-3p has been demonstrated in synovial fibroblasts in rheumatoid arthritis [310]. In another study, an overexpression of miR-323-3p has been shown to enhance activation of the Wnt pathway and to decrease the levels of its predicted target β-transducin repeat containing an inhibitor of beta-catenin [311]. The precise function of miR-323-3p and the whole cluster in immune system remains to be elucidated.

A growing body of data suggests that both Th17 cells and Th22 cells have important roles in allergic diseases. The Th17 cells have been demonstrated to be central in neutrophilic airway inflammation [290, 291]. IL-22 was shown to suppress recruitment of eosinophils and goblet cell hyperplasia in an antigen-induced airway inflammation in mice [289]. The levels of both IL-22 and IL-17 in serum of patients with asthma have been shown to be increased [312, 313]. In correlation with this, we currently observed the increased expression of miR-323-3p in PBMCs from patients with asthma. Further studies are needed to determine the role of miR-323-3p in IL-22/IL-17-producing T cells and asthma.

In conclusion, we demonstrated the increased expression of miR-323-3p in IL-22/IL-17-producing T cells of healthy individuals and in the PBMCs from patients with asthma, as well as the capacity of miR-323-3p to suppress IL-22 in T cells. These results suggest that miR-323-3p may influence T cell responses in asthma.

Acknowledgements

The authors thank Dr. Mark Ansel (Department of Microbiology & Immunology, University of California San Francisco, USA) for the detailed T cell transfection protocol, Dr. Eve Rannu for the careful selection of the patients with asthma and Mrs. Tiina Rebane for excellent technical assistance in preparing PBMCs.

Author contributions

J. K., M. W. and S. T. performed the experiments, analyzed the data, contributed with design of the study and writing the manuscript, T. R. performed the experiments and analyzed the data, A. Aints, K. Kisand, A. Altraja, K. Kingo, C. A. A. and M. A. contributed with design of the study and writing the manuscript, A. Altraja and K. Kingo organized collecting of peripheral blood samples, A. R. designed the study, wrote the manuscript and supervised the project. All authors have commented and approved the final version of the manuscript.

Funding

This work was supported by the Swiss National Science Foundation grants 32-132899, 32-140772, 32-159870 and 32-112306, the Christine Kühne-Center for Allergy Research and Education, Davos Switzerland (CK-CARE), Swiss-Polish contribution, institutional research grant IUT2-2, and personal research grants PUT214 and PUT177 from the Estonian Research Council, and the EU structural assistance grant SARMP12219T.

Figure legends

Figure 1 Human IL-22-positive T cells have distinct miRNA expression profile (A). Heatmap of the differentially expressed miRNAs in IL-22-positive T cells. Log2 expression values for each of the miRNAs are mean-centered across all samples for each gene separately. Color scale from blue (lower) to red (higher) represents deviation from the mean (black). (B) The relative levels of miRNAs with significantly different expression in IL-22-positive T cells. (C) Relative expression of the indicated miRNAs in IL-17-single positive, IL-22-single positive, IL-17- and IL-22-double positive, and IL-17- and IL-22-double negative cell populations. (B, C) Data represent the mean ± SEM of three independent healthy donors. Student's t-test, $*P < 0.05$, $**P < 0.05$

Figure 2 miR-323-3p inhibits genes from the TGF-β pathway and IL-22 production in human T cells. (A) The relative firefly luciferase (LUC) activity is compared to the control transfection (cont,=1), (n=6). (B-E) Purified CD3+ T cells were cultured 24h, transfected with pre-miR-146a (miR-146a), miR-323-3p or the control (cont) mimics, further cultured for 72h with an additional PMA/Ionomycin stimulation during last 6h. (B-D) The relative mRNA expression is shown compared to the average value in control transfected cells (cont,=1; n=7; cells were from 2 different healthy donors). (E) Quantification of IL-22 and IL-17 in the cell supernatants (n=4, cells from one representative healthy donor). (A-E) Data represent mean ± SEM, Student's t-test, $*P < 0.05$, $**P < 0.05$.

Figure 3 miR-323-3p is increased in PBMCs from patients with asthma. The relative expression of the miR-323-3p and miR-146a in PBMCs from patients with bronchial asthma (BA) and atopic dermatitis (AD) is shown in comparison with the control group. Student's t-test, $*P < 0.05$, $**P < 0.05$

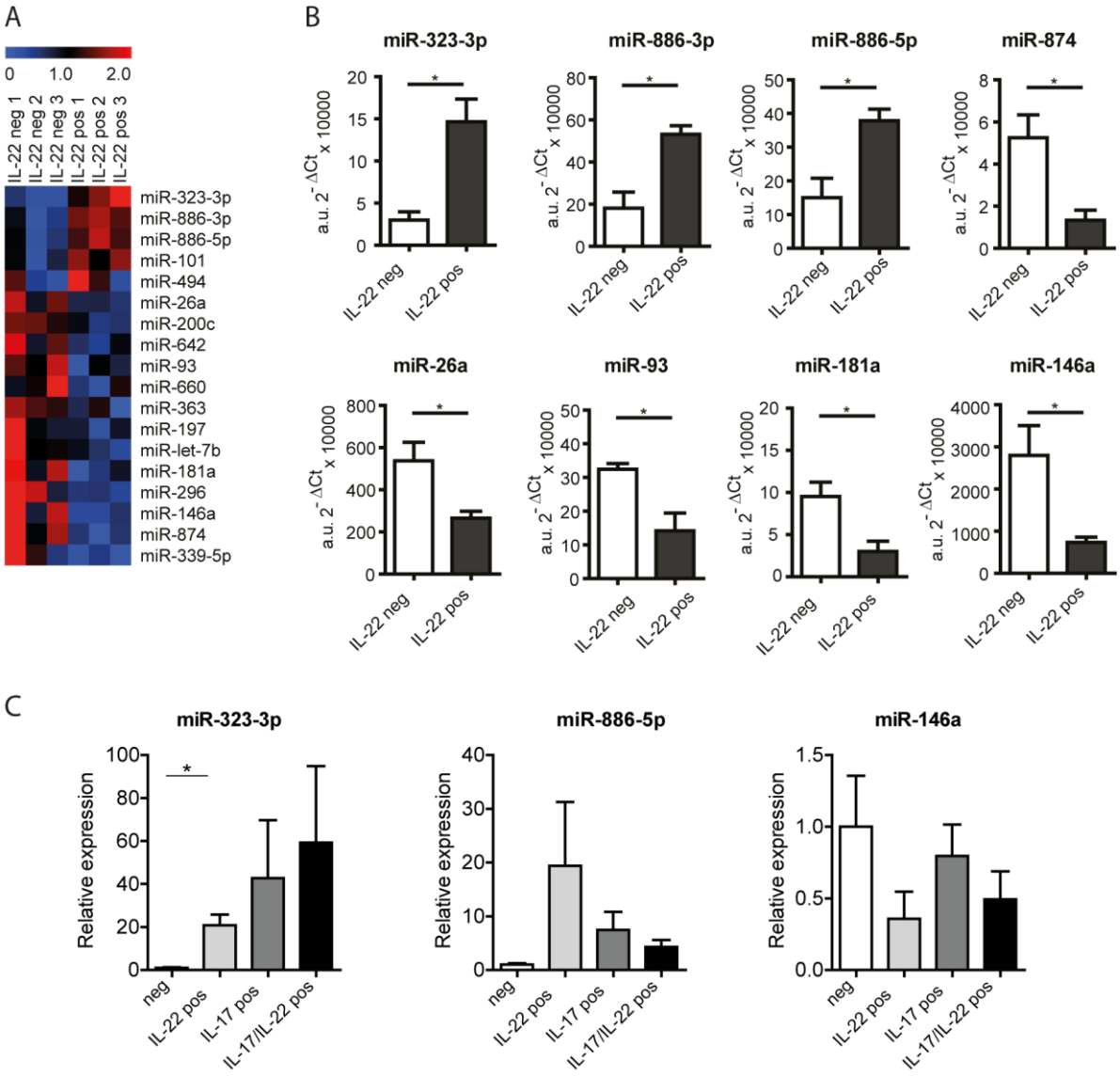

A
0
1.0
2.0
IL-22 neg 1
IL-22 neg 2
IL-22 neg 3
IL-22 pos 1
IL-22 pos 2
IL-22 pos 3
miR-323-3p
miR-886-3p
miR-886-5p
miR-101
miR-494
miR-26a
miR-200c
miR-642
miR-93
miR-660
miR-363
miR-197
miR-let-7b
miR-181a
miR-296
miR-146a
miR-874
miR-339-5p
B
miR-323-3p
miR-886-3p
miR-886-5p
miR-874
miR-26a
miR-93
miR-181a
miR-146a
a.u. 2-ΔCt x 10000
IL-22 neg
IL-22 pos
C
miR-323-3p
miR-886-5p
miR-146a
Relative expression
neg
IL-22 pos
IL-17 pos
IL-17/IL-22 pos

Figure 1

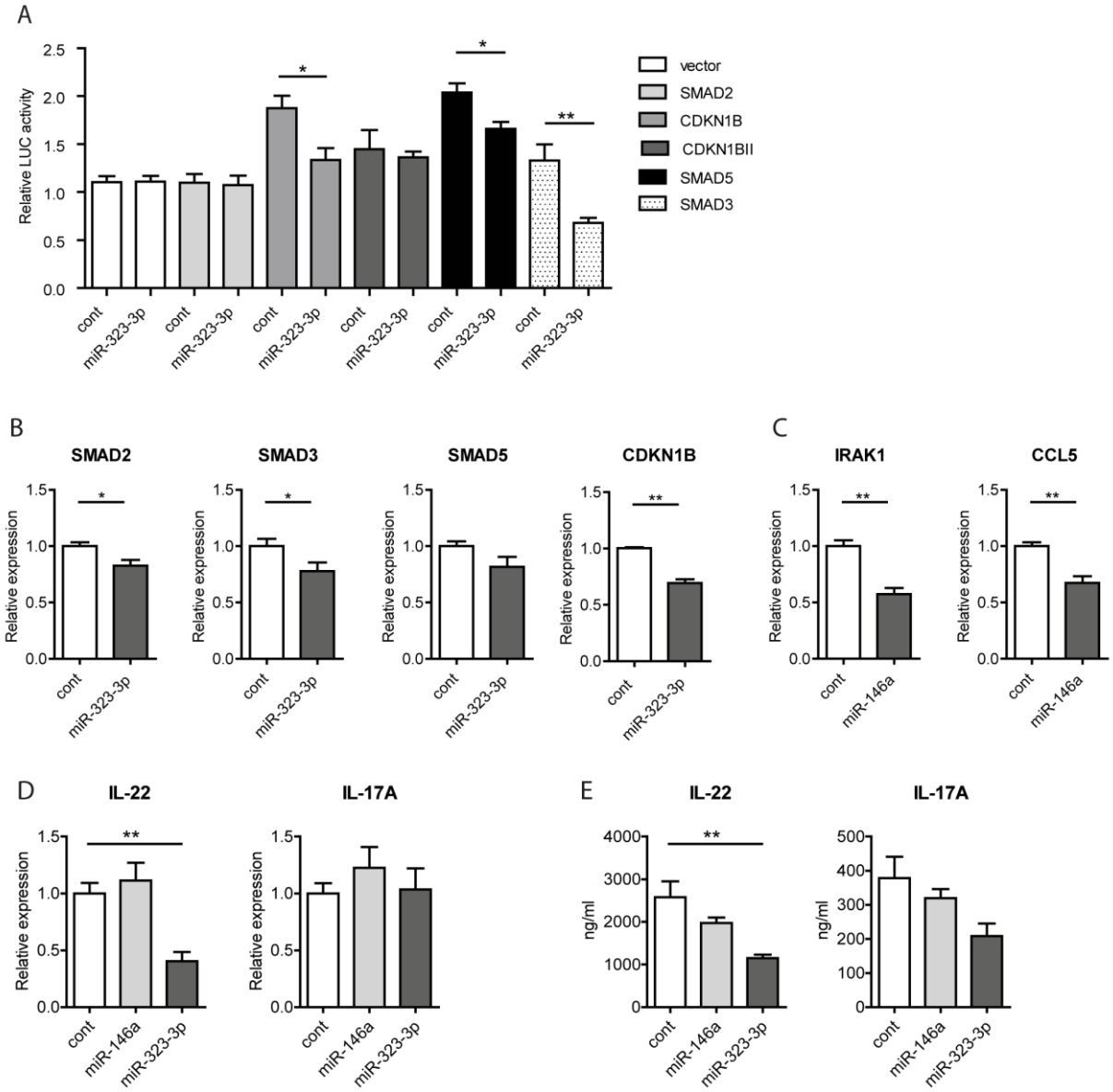

A
Relative LUC activity
vector
SMAD2
CDKN1B
CDKN1BII
SMAD5
SMAD3
cont
miR-323-3p
B
SMAD2
SMAD3
SMAD5
CDKN1B
C
IRAK1
CCL5
Relative expression
miR-146a
D
IL-22
IL-17A
E
ng/ml

Figure 2

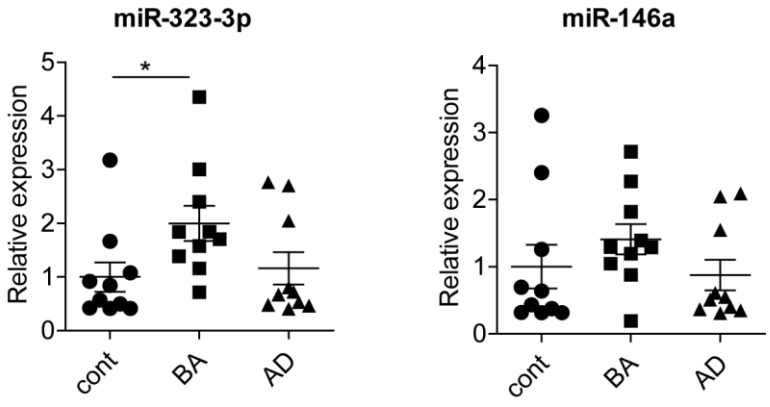

miR-323-3p
Relative expression
5
4
3
2
1
0
*
cont
BA
AD
miR-146a
Relative expression
4
3
2
1
0
cont
BA
AD

Figure 3

Table 1. miRNAs differentially expressed in IL-22-positive T cells potentially influence proliferation, survival and effector functions of T cells

miRNA	Significans[A]	Nr of targets	ID	Functional group an nr genes in the group		Putative targets in the functional group
miR-323-3p	1.04e-02	282	CORUM:3961	SMAD3-cSKI-SIN3A-HDAC1 complex	4	SIN3A,SKI,SMAD3
	4.89e-02	282	GO:0070848	response to growth factor stimulus	653	ARHGEF11,ARHGEF7,ATF1,CASP3,CCL2,CDKN1B,CHUK,EGR3,EIF2C1,ERBB4,FGF9,ITPR3,NLK,ONECUT2,PPP1CB,PPP1CC,PRDM4,RPS6KA3,SKI,SMAD2,SMAD3,SMAD5,TNRC6B
	1.26e-02	282	GO:0005057	receptor signaling protein activity	123	ARF1,CDKN1B,ERBB4,MAP3K2,MAP3K3,NLK,SMAD2,SMAD3,SMAD5,STK39
	1.30e-02	282	GO:0005072	TGF-beta receptor, cytoplasmic mediator activity	10	CDKN1B,SMAD2,SMAD3,SMAD5
miR-874	1.59e-02	392	GO:0034097	response to cytokine stimulus	692	ADAR,AFF3,BCL2,CD44,CDK9,IL2RB,IRF5,KPNA4,LEP,NUPL1,PIN1,RIPK1,SLC11A1,STAT2,STAT3,TPR
	6.79e-06	392	GO:0002252	immune effector process	761	ADAR,AP1S1,BCL2,BTN3A2,BTN3A3,CD247,CD59,CFL1,CLEC7A,CRK,CYFIP2,IRF5,ITPR2,MYO1C,NBN,POU2F2,RAB27A,SLC11A1,STAT2,TARBP2,TRIM25,VAV3
	3.82e-04	392	GO:0042110	T cell activation	453	AP3D1,BCL2,BTLA,BTN3A1,CD247,CD276,CD5,CD59,CLEC7A,MAP3K14,MYH9,PTPN22,RAB27A,SLC11A1,SMAD3
	1.81e-05	392	GO:0097190	apoptotic signaling pathway	473	ARHGEF6,BCL2,BMF,CD44,CD5,DYNLL1,DYRK2,HIPK2,MLLT11,PARK7,PPARD,RIPK1,SLC9A3R1,SMAD3,SORT1,TRAF1,VAV3
	2.10e-05	392	KEGG:04010	MAPK signaling pathway	289	ARRB1,CDC25B,CRK,DUSP22,IKBKB,MAP2K4,MAPK8IP3,NFATC1,PLA2G4C,PPM1A,STMN1,TAOK2
	1.11e-03	392	KEGG:04668	TNF signaling pathway	129	CREB1,CREB3L2,IKBKB,MAP2K4,RIPK1,TNFRSF1B,TRAF1
	7.69e-03	392	KEGG:04064	NF-kappa B signaling pathway	122	BCL2,IKBKB,PLAU,RIPK1,TRAF1,TRIM25
miR-93	3.91e-02	317	GO:0045321	leukocyte activation	719	BCL2,CBLB,CHD7,PAG1,PDPK1,PRDM1,PRKCD,PTPRE,RORA,ZFP36L1,TNFSF11,TNFSF4,TXK,YWHAZ
	2.32e-03	317	GO:0097191	extrinsic apoptotic signaling pathway	153	BCL2,CYLD,DDX3X,ITGA6,MADD,MCL1,PDPK1,PHIP
	7.09e-03	317	KEGG:04010	MAPK signaling pathway	289	DUSP5,MAP2K1,MAP3K3,MAP4K4,MKNK2,NLK,RPS6KA3,TAOK1
	2.20e-02	222	GO:0001816	cytokine production	599	ATG12,CSF1R,IQGAP1,IRAK4,ITCH,RAC1,RASGRP1,RNF135,SLC11A1,SMAD4,TRAF6
miR-146a	5.61e-09	222	GO:0042981	regulation of apoptotic process	1344	ATG7,BCLAF1,BTG2,CASP7,CTSB,DYNLL2,EDNRB,ERBB4,IFIT3,ITCH,ITGAV,MTDH,NRAS,PMAIP1,PSMC6,PSMD3,PSMF1,RAC1,RHOA,RRM2B,SIAH2,SIN3A,STIL,STK3,TRAF6,USP47
	3.14e-02	222	KEGG:04010	MAPK signaling pathway	289	NRAS,PPM1A,RAC1,RASGRP1,STK3,TRAF6
miR-26	3.25e-02	231	GO:0042127	regulation of cell proliferation	1371	MAB21L1,HMGA1,NAMPT,SCN5A,HOXD13,CDK13,NACC2,CTGF,GDF11,SMAD4,HAS2,TP63,TCF7L2,LHX1,EREG,KDM4C,FGF21
	5.91e-03	231	GO:0000165	MAPK cascade	599	ATF2,PRKCD,ADAM9,VANGL2,PLCB1,TRIB2,TAB3,LPAR3,DAB2,CTGF,FGF14,FGF21
	1.29e-02	231	KEGG:04310	Wnt signaling pathway	150	VANGL2,PLCB1,FRAT2,SMAD4,TCF7L2

[A] Significance designates the P value from Fisher exact test showing the significance of the overlap between the target list and the indicated functional category.

Table 2. Predicted miR-323-3p binding sites in the genes from the TGF-β pathway

Position of miR-323-3p[A]	miRNA and mRNA sequences	Total context score[B] and conservation[C]
310-317 nt of SMAD2 3' UTR	5' ...UAGUUAUGUGUACAGGUAAUGUA... ||||||| 3' UCUCCAGCUGGCACAUUACAC	Conserved, -0.2
3980-3987 nt of SMAD3 3' UTR	5' ...UUUUUUAAUGCAGAAGUAAUGUA... ||||||| 3' UCUCCAGCUGGCACAUUACAC	Conserved, -0.17
627-633 nt of SMAD5 3' UTR	5' ...UAAGUGUUCUUCAGUGUAAUGUG... ||||||| 3' UCUCCAGCUGGCACAUUACAC	Conserved, -0.07
1274-1280 nt of CDKN1B 3' UTR	5' ...CAAGUAUAAUUUUUUGUAAUGUG... ||||||| 3' UCUCCAGCUGGCACAUUACAC	Conserved -0.24
439-445 nt of CDKN1B 3' UTR	5' ...GUUUUCUUUAAAGAUGUAAUGUC... ||||||| 3' UCUCCAGCUGGCACAUUACAC	Unconserved -0.24

[A]Positions are shown from the first nt of the corresponding human gene 3'UTR

[B]Conserved = conserved across most of the mammals, but usually not beyond placental

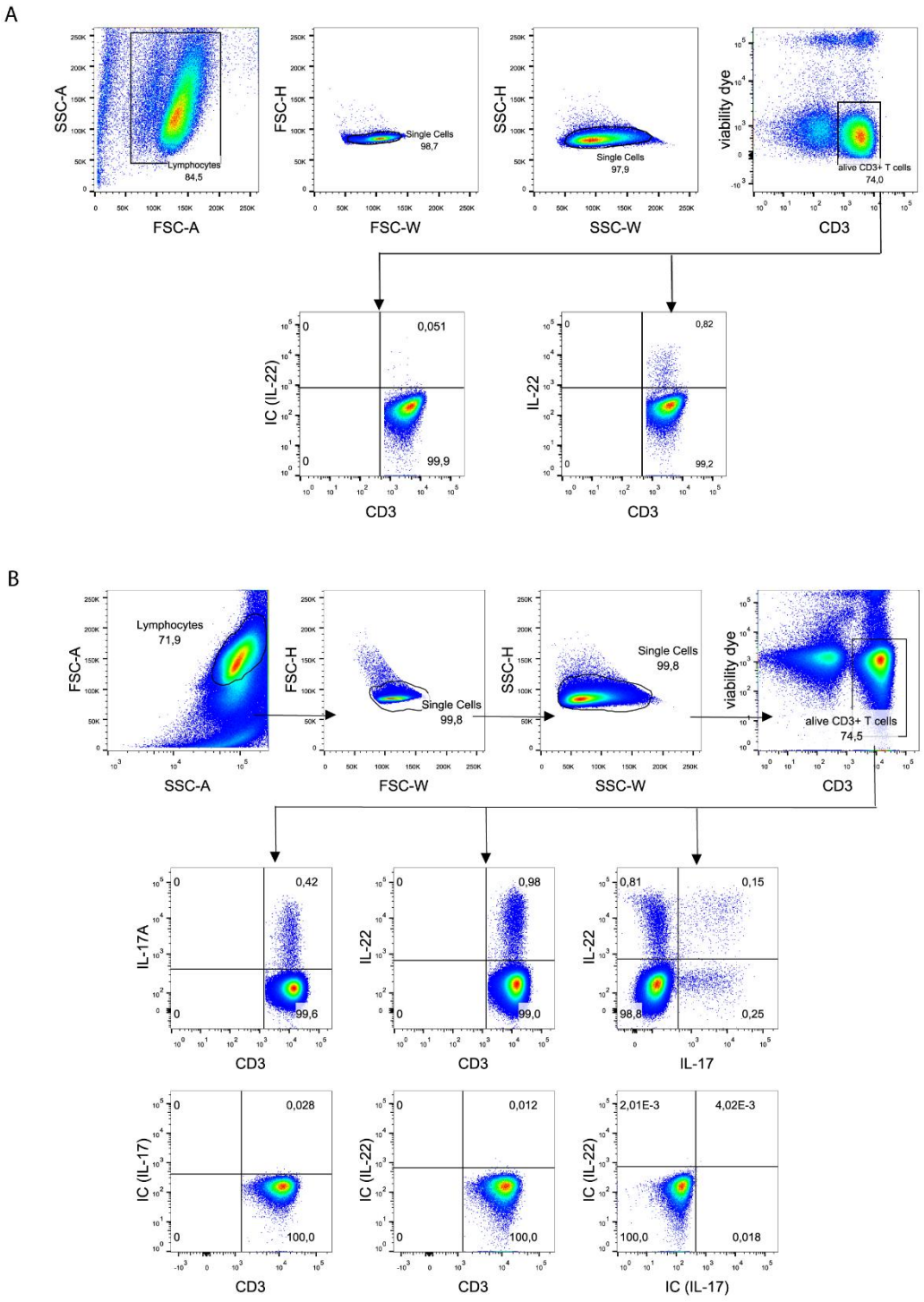
[C]The context+ score for a specific site is the sum of the contribution of six features, including site-type, 3' pairing, local AU, position, target-site abundance and seed-pairing stability contributions.

Supporting figure legends

Figure S1 Sorting strategy for the IL-22- and IL-17-positive T cells. Gating strategy for sorting of IL-22-producing T cells (A) and IL-17- and IL-22-single or double-producing T cell-subsets (B). Based on the forward scatter (FSC) and side scatter (SSC) characteristics of the cells, the lymphocyte population was included in the analysis. After doublet cells exclusion using height (FSC-H, SSC-H) and width (FSC-W, SSC-W) of the peak, viable CD3+ T cells producing IL-22 and not producing IL-22 were sorted (A). The very same strategy of gating was applied to sort IL17/IL-22-positive ($CD3^+IL\text{-}17^+IL\text{-}22^+$), IL-17-positive ($CD3^+IL\text{-}17^+IL\text{-}22^-$), IL-22-positive ($CD3^+IL\text{-}17^-IL\text{-}22^+$), and double negative ($CD3^+IL\text{-}17^-IL\text{-}22^-$) T cells. Quadrant gates were applied based on the isotype control (IC) staining.

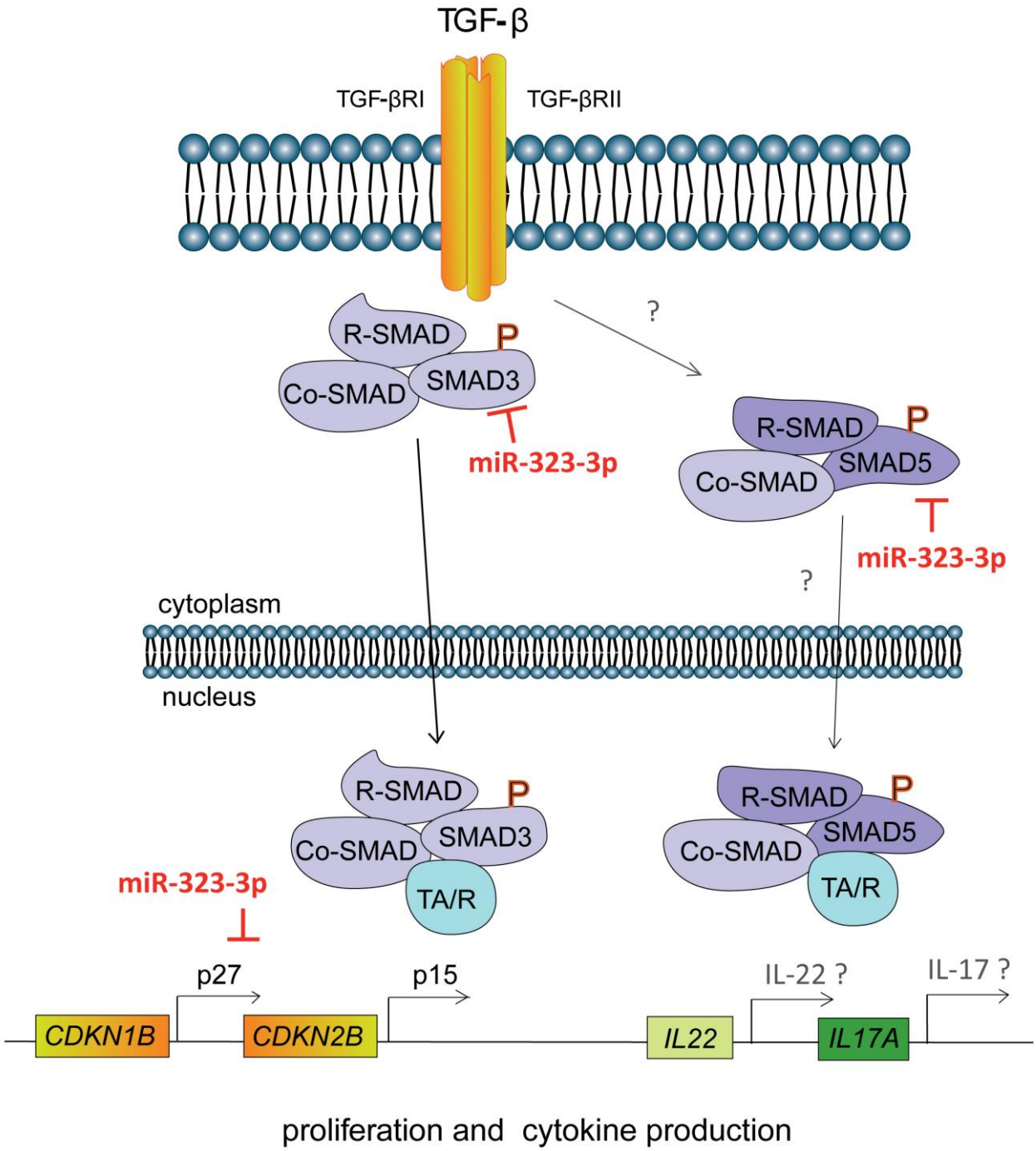
Figure S2 Proposed model of miR-323-3p action in the IL-17/IL-22-positive T cell-subset. The presence of TGF-β is needed for the development of functional Th17 cells, but at the same time it generally counteracts the production of IL-22. The binding of TGF-β induces the assembly, auto-phosphorylation and activation of the type I and type II TGF-β receptors, followed by phosphorylation of R-SMAD (receptor-regulated SMAD, mothers against decapentaplegic Homologue)) proteins [305]. SMAD3 is a R-SMAD protein from the canonical TGF-β pathway. SMAD5 is a R-SMAD protein that participates in signaling mediated by the bone morphogenic protein (BMP) (not shown in the figure) [305] and possibly by TGF-β [314]. Phosphorylated R-SMADs form homo-oligomeric and hetero-oligomeric complexes with the co-mediator SMAD (Co-SMAD). These complexes are translocated into the nucleus, where they associate with transcriptional activators or repressors (TA/R) to regulate the transcriptional activity of numerous genes [305]. miR-323-3p targets the TGF-β pathway through direct targeting of SMAD3, SMAD5, and cyclin-dependent kinase inhibitor 1B (CDKN1B, also known as p27) and thereby also modulates the expression of other regulators of the cell cycle, including CDKN2B (also known as p15) and cell proliferation. In addition, the increased expression of miR-323-3p inhibits the production of IL-22.

.



Supplementary figure 1

.



Supplementary figure 2

6.2 Transcriptomic characterization of human IL-22-producing T cells by next generation RNA sequencing.

Wawrzyniak M. [a], *Wawrzyniak P.* [a,b], *Breedveld A.* [a], *Rebane A.* [c], *Morita H.* [a], *Rückert B.* [a], *Castro Giner F.* [a], *Neumann Avidan.* [a], *Rhyner C.* [a], *Akdis C.A.* [a,b], *Akdis M.* [a,]

[a] Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland.

[b] CK-CARE AG - Christine Kühne – Center for Allergy Research and Education, Davos, Switzerland

[c] Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia.

Corresponding author:

Akdis Mübeccel,

Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Obere Strasse 22, Tel.: +41 81 410 08 48, akdism@siaf.uzh.ch

Acknowledgments

This work was supported by the Swiss National Science Foundation grants 32-132899, 32-140772, 32-159870 and 32-112306, the Christine Kühne-Center for Allergy Research and Education, Davos Switzerland (CK-CARE), Swiss-Polish contribution, institutional research grant IUT2-2.

Word count: 1489

Capsule Summary

CD4+ T cells differentiate into effector T cells upon antigen recognition during immune synapse formation and delivery of three signals by antigen-presenting cells. The main subsets of effector T helper cells are: Th1, Th2, Th9, Th17 and Th22 cells. Among all T helper cell subsets, IL-22-producing T cells have been characterized to a lesser extent as compared to other subsets. The present study for the first time shows next generation RNA sequencing analysis of human IL-22-producing T cells and proposes the genes that are describing in detail human IL-22-producing T cells. However, further investigation of the biological function of these molecules in IL-22 secreting T cells is required. Additionally, there is certainly a need to distinguish between IL-22-producing Th17 cells and IL-22-producing Th22 cells and better characterize these pure human Th22 cells.

Key Words

Next generation sequencing, Th22, Th17, IL-17A, IL-22, cytokines, transcription factors,

To the Editor:

T cells mediate adaptive immune responses via the activation of other immune cells and the regulation of tissue cells to eliminate bacterial, fungal and viral pathogens and maintain homeostasis. Based on cytokine production patterns, T helper cells are classically subdivided into Th1, Th2 and T regulatory cells [315]. Recently, new subsets of T helper cells, including Th9, Th17 and Th22 cells have been described [89, 147]. The main cytokine produced by Th17 and Th22 cells is interleukin-22 (IL-22) [316]. Because of the preferential expression of IL-22R1 on cells of epithelial origin, IL-22 is a unique cytokine produced by immune cells and acts only on tissue cells [127]. The effects of IL-22 on tissue cells can be grouped into five main categories. First of all, IL-22 influences terminal differentiation of keratinocytes [171]. Secondly, IL-22 via the induction of antibacterial protein production by epithelial cells increases the innate immune defense mechanisms against microbes [169]. Additionally, it enhances the production of chemokines by epithelial cells and the migratory ability of epithelial cells [169, 172]. Finally, by the upregulation of STAT3, IL-20 and IL-22 show a self-perpetuating action on epithelial cells [317, 318]. On the contrary, when it is uncontrolled, IL-22 may lead to inflammation that can be seen in psoriasis [317], atopic dermatitis [177] or rheumatoid arthritis [197]. As compared to other cytokine-producing cells, IL-22-producing T cells are poorly characterized so far. One of the possible reasons may be due to the lack of a key transcription factor responsible for induction of IL-22 production.

Because of unique role of IL-22 in the regulation of epithelial cells, the characterization of IL-22-producing T cells was performed in human palatine tonsils, where T cells are in close proximity to epithelial cells. In the present study, viable human IL-22-producing T cells from palatine tonsils have been isolated with the use of our newly generated IL-22-secretion assay and next generation RNA sequencing transcriptome analysis has been performed on these cells.

First of all, the general characterization of tonsillar T cells in terms of the production of the main Th1/Th2/Th17/Th22-derived cytokines (IL-22, IL-17A, IL-10, IL-4, IL-13 and IFN-γ) was assessed in healthy as well in allergic donors by intracellular cytokine staining after PMA and ionomycin stimulation (Fig. 1 A-C) (Suppl. Figure 1A). There was no difference in cytokine production including IL-22 between healthy and allergic donors (Figure 1A). In both groups, the percentage of $CD4^+IL\text{-}17^+$ cells was higher compared to $CD4^+IL\text{-}22^+$, $CD4^+IL\text{-}4^+$ and $CD4^+IL\text{-}13^+$ T cells. Moreover, the percentage of $CD4^+IL\text{-}10^+$, $CD4^+IL\text{-}4^+$ and $CD4^+IFN\text{-}\gamma^+$ cells was higher than the percentage of $CD4^+IL\text{-}13^+$ cells in both groups (Suppl. Figure 1B). Next, the co-expression analysis of IL-17, IL-10, IL-4, IL-13 and IFN-γ by IL-22-producing $CD4^+$ T cells revealed that most of the IL-22-producing cells either co-express IL-4 only or co-express IL-17 only or produce IL-22 alone (Figure 1B). Interestingly, when a similar analysis was performed for IL-17-producing, IL-4-producing, IFN-γ-producing and IL-10-producing T cells, the percentages of single IL-17-positive, single IL-10-positive, single IL-4-positive or single IFN-γ-positive cells were statistically higher when compared to IL-22-single producing T cells (Figure 1C, Suppl. Figure 1C). The results mentioned above support the common understanding that IL-22 is a bifunctional cytokine, which may play both protective and pro-inflammatory roles on epithelial cells depending on cytokine environment.

Furthermore, we investigated which stimuli were optimal for the induction of IL-22 production by T cells. Firstly, tonsil mononuclear cells (TMC's) have been stimulated with all Toll-like receptor ligands and Poly (I:C), a TLR3 ligand was found to induce IL-22 production from TMC`s (Figure 1D). To clarify which cells are responsible for IL-22 production, T cells have been isolated from TMC`s and stimulated with this TLR3 ligand in the presence of IL-7 and IL-23 (Figure 1F). A high induction of IL-22 production from those

cells indicated that the stimulation with TLR3L, IL-7 and IL-23 is optimal for IL-22 induction from T cells.

To date, the only method to analyze IL-22-producing cells is cytokine intracellular staining in which the permeabilization - fixation protocol leads to cell death, therefore these cells are not suitable for culture and detailed analysis. To overcome this problem, an in-house IL-22-secretion assay has been developed (Suppl. Figure 1A).

Cells stimulated with the combination of TLR3 ligand, IL-7 and IL-23 have been sorted with this IL-22 secretion assay and mRNA expression for IL-17, IL-22 and IFN-γ was measured. The analysis of mRNA for cytokines shown in figure 1G suggest that it is possible to detect and successfully sort the IL-22-producing T cells that are characterized by the high expression of IL-22. Furthermore, sorted IL-22-positive and IL-22-negative T cells have been cultured in the presence of common gamma chain receptor cytokines (Suppl. Figure 2B, Suppl. Figure 2C). Two days after sorting, and during the long-term culture, sorted cells retain their initial cytokine profile suggesting that the IL-22-secretion assay is a reliable tool for the isolation of IL-22-producing T cells.

Next generation RNA sequencing analysis of IL-22-producing and IL-22-non-producing T cells has been carried out. Differential gene expression analysis revealed that 57820 genes are differentially expressed in IL-22-producing T cells as compared to IL-22-negative T cells. To narrow down the number of genes: genes only expressed in 3 out of 4 donors in either IL-22-positive or IL-22-negative T cells with the intensity values above 0, with a log2 value ratio ≥ 1 or ≤ -1 and differently expressed with the p-value below 0.01 has been included in the analysis. A list of 601 genes fulfilling the above mentioned criteria has been analyzed with the help of Metacore – data mining and pathway analysis software. 13 genes recognized as coding receptor ligands, 13 genes as coding transcription factors and 6 genes as chemokine receptors have been

identified within the list as differentially expressed between IL-22-positive and IL-22-negative T cells (Figure 2, Suppl. Figure 3).

Signature genes characterizing IL-22-producing T cells may be subdivided into 3 main groups: cytokines and chemokines, G protein-coupled receptors (GPCRs) and transcription factors (TFs). Among the cytokines, IL-22-producing T cells express higher levels of IL-22, IL-17A, IL-17F, IL-1A, IL-9, IL-21, IL-26 and CCL1, CCL2, CCL20 (Figure 2A). As IL-17A and IL-17F production by IL-22-positive T cells have been already described, other molecules are reported for the first time to be co-expressed together with IL-22. Interestingly, IL-9, which is secreted by IL-22-producing cells acts on variety of cells including mast cells, T regulatory cells, Th17 cells and antigen presenting cells [319]. Furthermore, IL-26, a member of the IL-10 cytokine family acts mainly on monocytes [320]. Interestingly, IL-26 was found to kill extracellular bacteria via membrane-pore formation. IL-26 forms complexes with prokaryotic and eukaryotic DNA that trigger the production of type I interferon by pDCs [321]. IL-21 induces IL-22 production from T cells and may serve as a positive feedback loop [322]. Additionally, CCL1 has been indicated to be involved in Th2 and Treg cell trafficking, CCL2 has been reported in inflammatory monocyte trafficking and CCL20 in Th17 responses [323]. Collectively, this data suggests that IL-22-producing T cells may influence the tissue responses and at the same time through the production of other cytokines and chemokines may influence immune cells and exhibit a broader effect on the immune system. As IL-22 is known to be bi-functional, depending on the cytokine milieu [324], it remains unclear what would be the functional effect of IL-22 on the immune cells in the context of the above mentioned co-produced cytokines. At the same time, IL-22-producing T cells differentially express CCR5, CXCR4 and CXCR6 that may be used as surface markers of IL-22-producing cells (Figure 2B). Interestingly, CCR5 is described as an inflammatory chemokine receptor [323]. CCR5 is expressed by Th1 cells, however more is known about the expression

of CCR5 on Treg cells, which is critical for its migration to tissues infected with fungal pathogens [325] and *Leishmania major* [326]. Additionally, during graft-versus-host disease, CCR5 mediates the accumulation of Treg cells in the graft-versus-host disease target organs [327]. In the islet allograft transplant model, Treg migration from the blood to inflamed transplant tissue requires CCR5 expression [328]. A similar role in relation to migration of cells to tissue was found for CXCR6 which promotes NKT cells residence in peripheral tissues like liver and lung [329]. In contrast, another chemokine receptor expressed by IL-22-positive T cells - CXCR4, mediates CCR7-independent entry to the lymph nodes [330]. It is possible that chemokine receptors expressed differentially on IL-22-positive cells may guide the migration of these cells to inflamed tissues where they may influence tissue epithelial cells via the production of IL-22.

Finally, among potential transcription factors upregulated in IL-22-producing T cells, - PPARG and among transcription factor downregulated in IL-22-producing T cells - FOS, bind directly to the IL22 promotor and may influence the IL-22-production directly [SABiosciences` proprietary database] (Figure 2C). The role of other transcription factors upregulated (MAF, EPAS1, MSC, CITED) and downreagulated (EGR1, EGR4, ZXDA) in IL-22-positive T cells has to be investigated in more details.

In summary, the present study, for the first time shows the next generation RNA sequencing analysis of human IL-22-producing T cells and proposes the genes that can characterize human IL-22-producing T cells in detail. Furthermore, the investigation of biological function of these molecules in IL-22-secreting T cells is required. Additionally, there is a need to distinguish between IL-22-producing Th17 cells and IL-22-producing Th22 cells to better characterize pure human Th22 cells.

Figure legends

Figure 1. Characterization of human IL-22-producing T cells.

Tonsil mononuclear cells from healthy and allergic individuals were stained intracellular for IL-22, IL-17A, IL-4, IL-13, IL-10 and IFN-γ. The expression of cytokines was analyzed in viable CD3+CD4+ T cells (A). Co-expression of IL-17A, IL-4, IL-13, IL-10 and IFN-γ by CD3+CD4+IL-22-producing T cells was evaluated (n=7) (B). IL-17A, IL-4, IL-13, IL-10 and IFN-γ-producing T cells and cells expressing a single cytokine of interest that do not express other cytokines has been shown (C). To investigate the optimal culture conditions to induce IL-22 production, TMCs from healthy donors were stimulated with a combination of TLR3 ligand (Poly I:C), IL-7 and IL-23 and mRNA expression of IL-22 has been analyzed (n=3) (D). IL-22 protein levels in the culture supernatants of TMCs (E) and T cells isolated from TMC`s (F) stimulated in the presence of TLR3 ligand IL-7 and IL-23 was measured (n=4). (G) Relative mRNA expression of IL-22, IL-17 and IFN-γ by IL-22-positive and IL-22-negative T cells sorted with IL-22-secretion assay is shown (n=3).
(C) One-way Anova test with multiple comparisons was calculated (*** $p<0.0005$).
(F) Mann Whitney test was applied (* $p<0.05$).

Figure 2. Next generation sequencing characterization of human IL-22-producing T cells.

Reads per kilo base per million (RPKM) values of differentially expressed genes between IL-22-positive over IL-22-negative T cells has been plotted for cytokines and chemokines related genes (A), chemokines receptors genes (B) and transcription factors genes (C) (n=4).

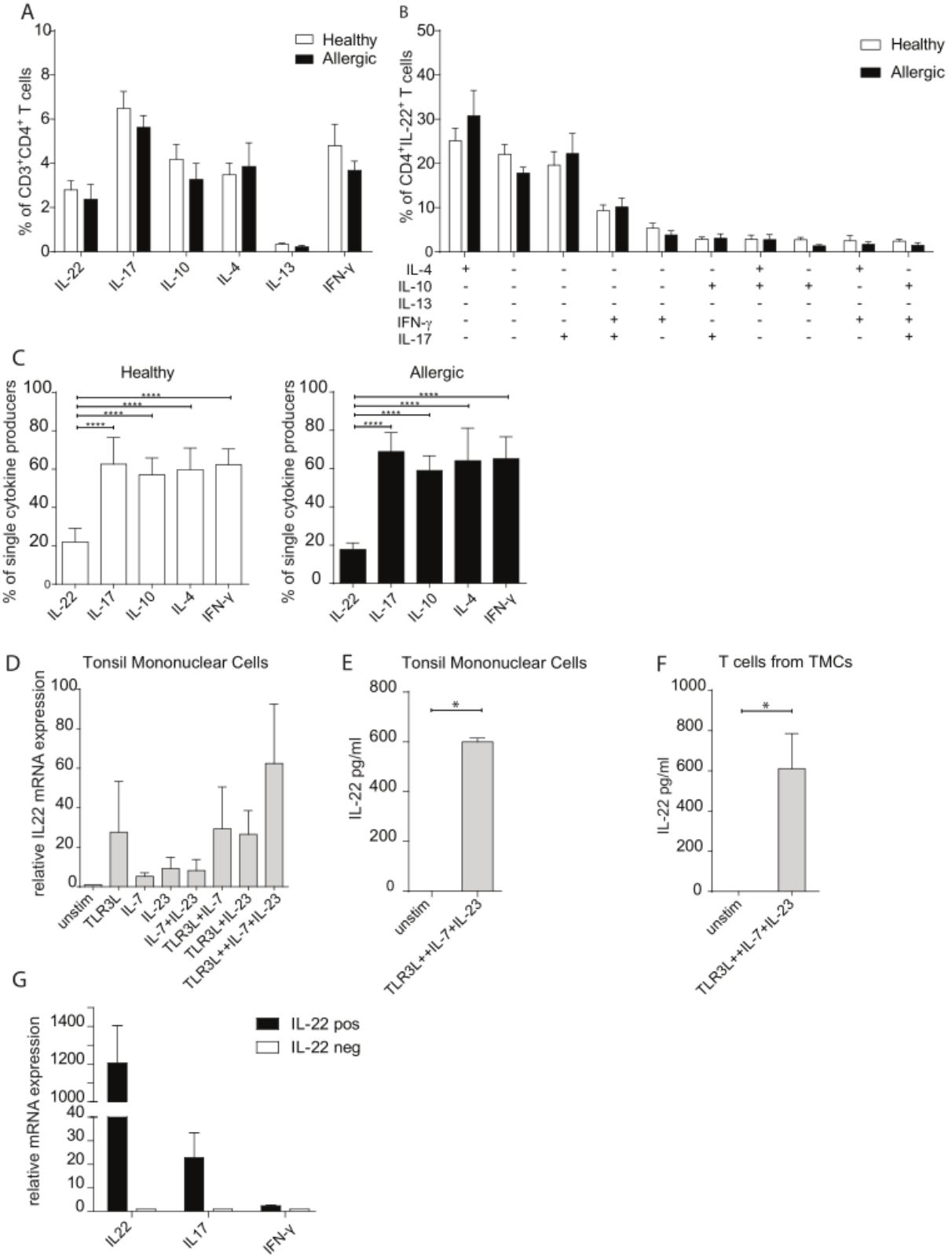

A
% of CD3+CD4+ T cells
Healthy
Allergic
IL-22
IL-17
IL-10
IL-4
IL-13
IFN-γ
B
% of CD4+IL-22+ T cells
Healthy
Allergic
IL-4
IL-10
IL-13
IFN-γ
IL-17
C
Healthy
Allergic
% of single cytokine producers
IL-22
IL-17
IL-10
IL-4
IFN-γ
D
Tonsil Mononuclear Cells
relative IL22 mRNA expression
unstim
TLR3L
IL-7
IL-23
IL-7+IL-23
TLR3L+IL-7
TLR3L+IL-23
TLR3L++IL-7+IL-23
E
Tonsil Mononuclear Cells
IL-22 pg/ml
unstim
TLR3L++IL-7+IL-23
F
T cells from TMCs
IL-22 pg/ml
unstim
TLR3L++IL-7+IL-23
G
relative mRNA expression
IL-22 pos
IL-22 neg
IL22
IL17
IFN-γ

Figure 1

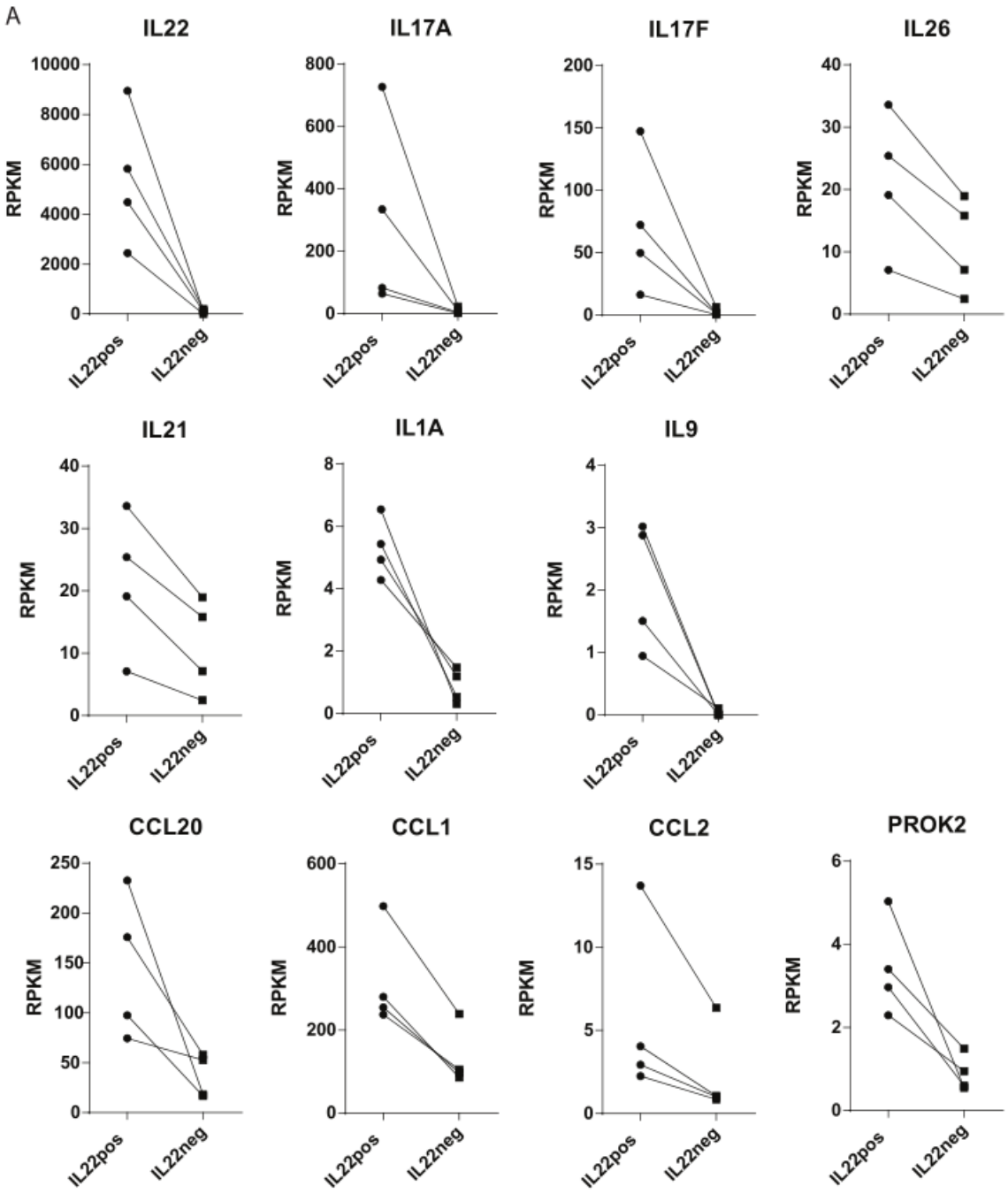

A
IL22
IL17A
IL17F
IL26
IL21
IL1A
IL9
CCL20
CCL1
CCL2
PROK2
RPKM
IL22pos
IL22neg

Figure 2A

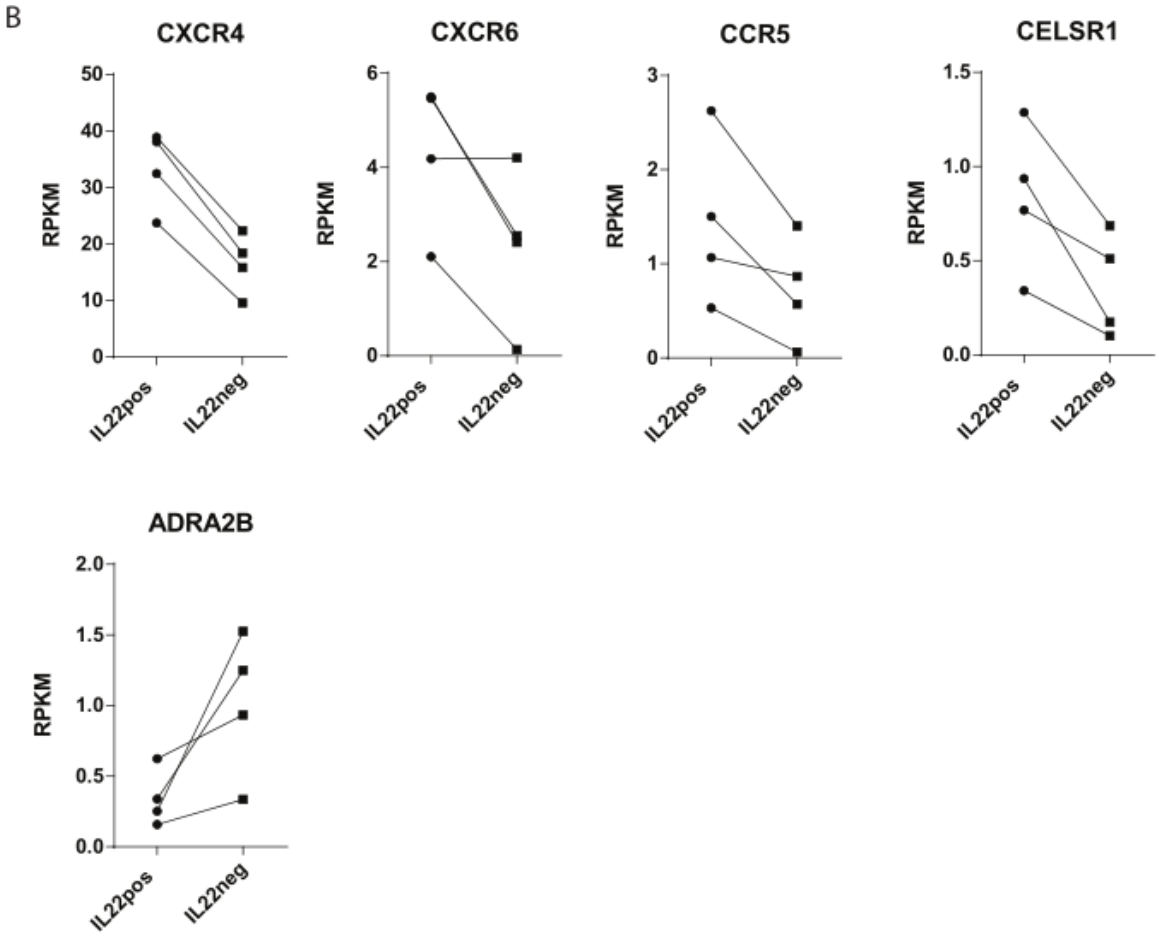


Figure 2B

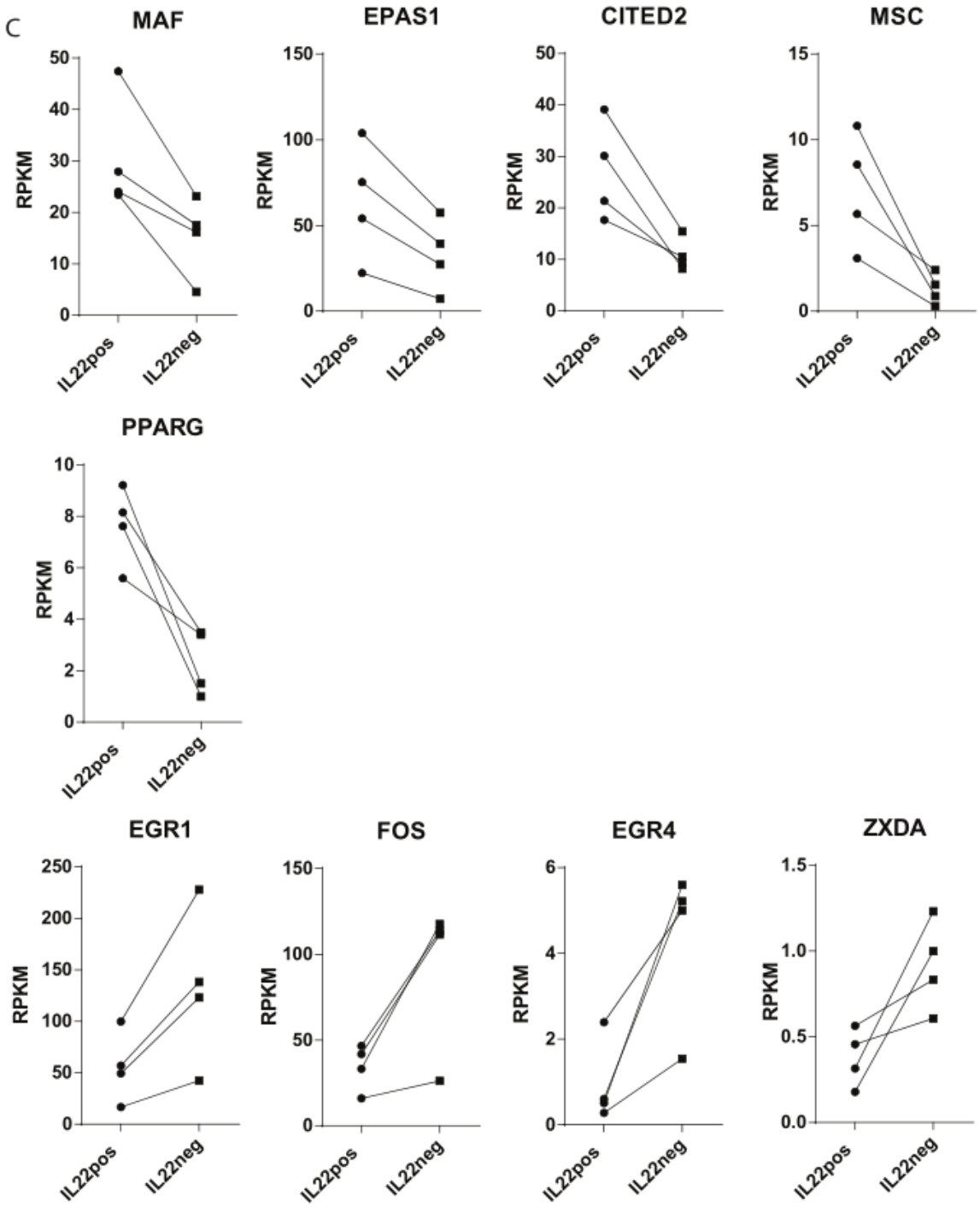

C
MAF
EPAS1
CITED2
MSC
PPARG
EGR1
FOS
EGR4
ZXDA
RPKM
IL22pos
IL22neg

Figure 2C

Online Repository Figure Legends:

Supplementary figure 1. Cytokines intracellular staining characterization of human tonsillar T cell.

For the gating strategy for intracellular staining of cytokines produced by T cells in tonsil mononuclear cells, firstly, cell doublets were discriminated. Later only viable, CD3+CD4+ positive T cells were included and every cytokine was plotted against the CD4 marker to show the percentage of cytokine-producing CD3+CD4+ T cells (A). The comparison of cytokine-producing CD3+CD4+ cells in healthy and allergic donors was analyzed (B). Co-production of other cytokines by cytokine-producing T cells were analyzed using the TREE function of KALUZA software which allows to calculate all possible combinations of coproduction of 5 cytokines by specific cytokine-producing cells. In this analysis, 100% of cells are considered as all cells that are able to produce specific cytokine (C). One-way Anova test with multiple comparisons was used (* $p<0.05$,** $p<0.005$,*** $p<0.0005$).

Supplementary figure 2. T cells sorting with IL-22-secretion assay.

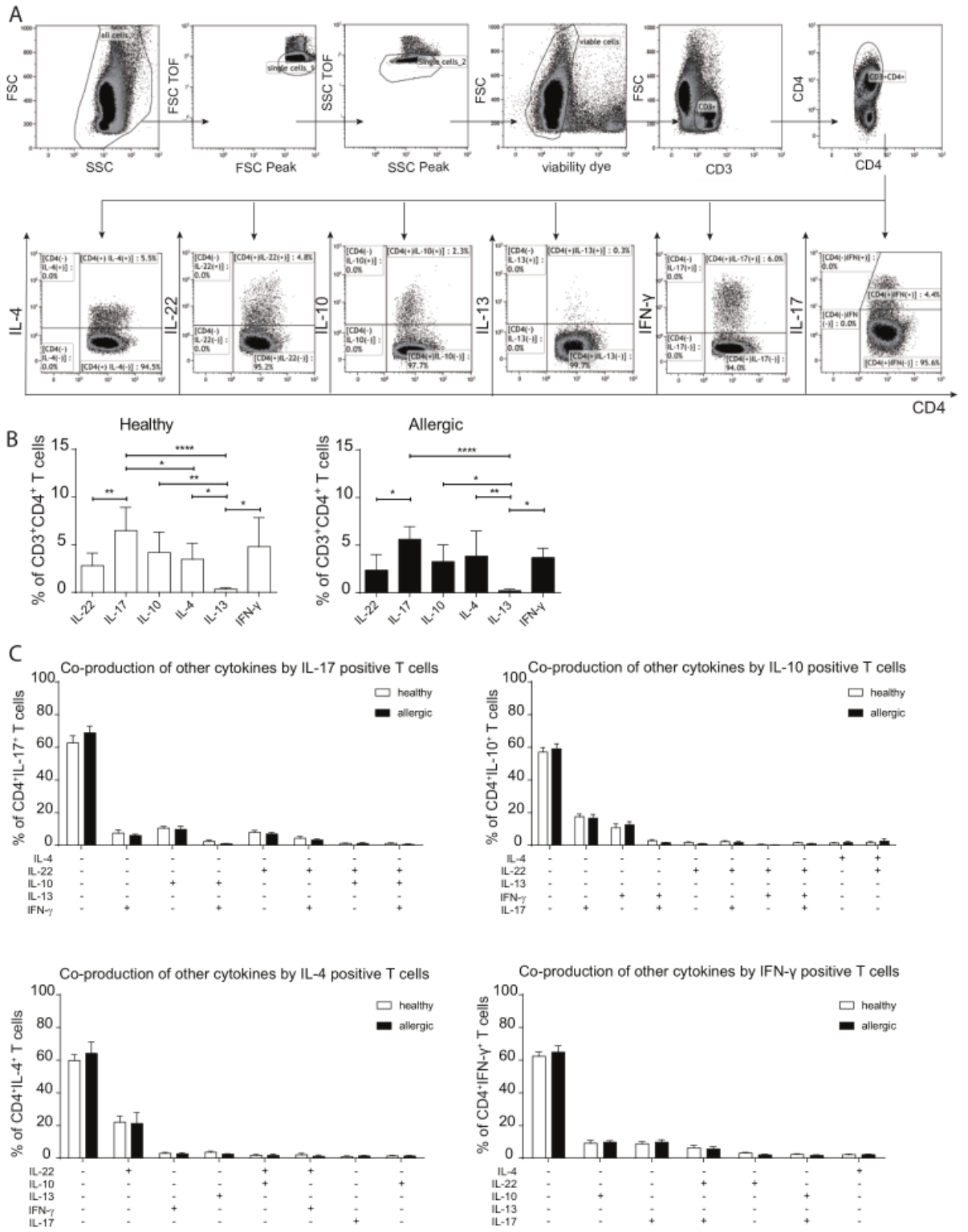
Gating strategy for sorting of IL-22-positive and IL-22-negative CD3+CD4+ T cells from tonsils (A). Gates defining the positive population were set up based on a control that did not contain anti-IL-22-catch reagent (A). In both sorted populations mRNA expression of common gamma chain receptor family receptors has been analyzed (B) and sorted IL-22-positive and IL-22-negative cells have been cultured in the presence of IL-2 and the profile of cytokine production was assessed on day 2 and day 7 after second re-stimulation with anti-CD3, anti-CD2 and anti-CD28 monoclonal antibodies.

Supplementary figure 3. Validation of anti-IL-22-catch reagent formation.

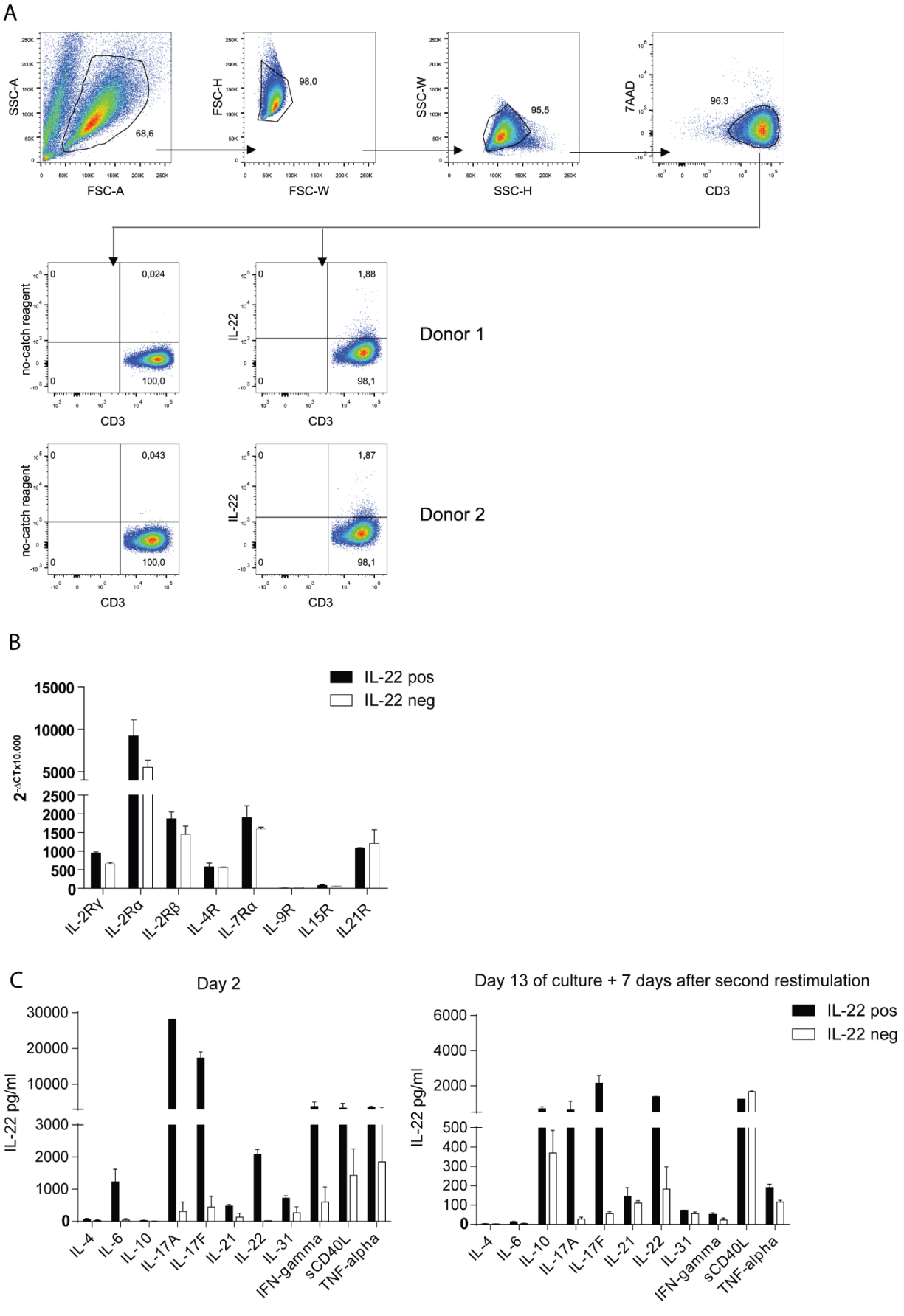
IL-22 secretion assay consists of anti-IL-22 catch reagent and anti-IL-22 detection antibody. Anti-IL-22 catch reagent is formed by cross linking mouse anti-human CD45 Ab with rat anti-human IL-22 Ab. Rat anti-human IL-22 antibody which is the part of anti-IL-22 catch reagent was detected with goat-anti rat AF488 Ab to prove that anti-IL-22 catch reagent is present on the cells and that catch reagent was formed properly. Different dilutions of catch reagent were added to stain $1x10^6$ (A) or $10\ x10^6$ (B) cells, ranging from undiluted to 10, 50, 100 x diluted.

Supplementary figure 4. Next generation sequencing characterization of human IL-22-producing T cells.

Volcano plots for genes upregulated and downregulated in IL-22-positive versus IL-22-negative sorted T cells (-log10 FDR vs log2 ratio) (A). Heat-map for clustering with GO categories for only those genes expressed in at least 75% of samples in either or both samples (B). A heat-map normalizing the expression values by pair and sorting samples by patients for all GO-categories (C) and for GO immune system processes genes (D).

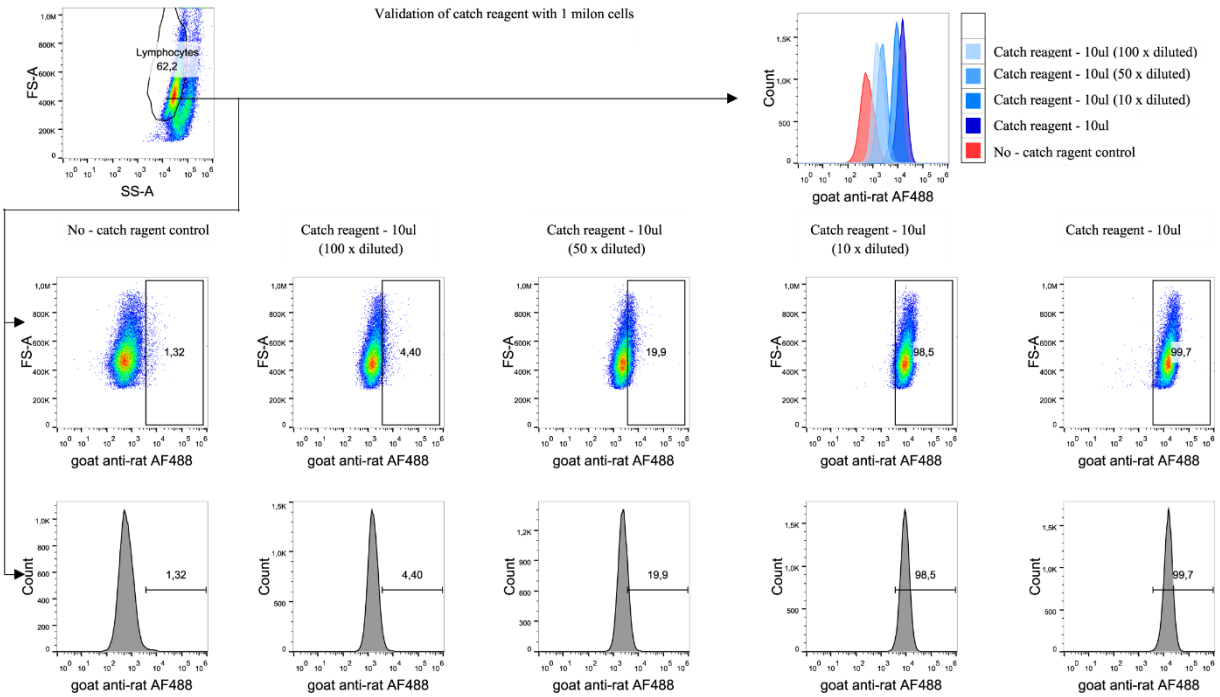


Supplementary figure 1

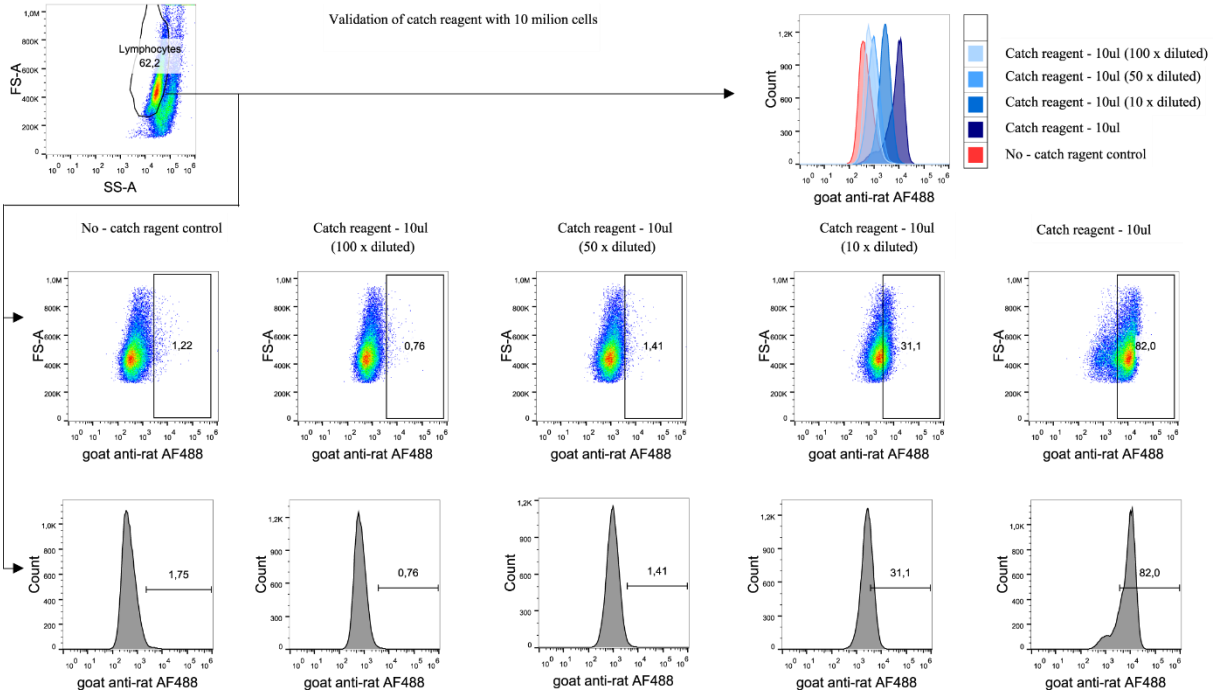


Supplementary figure 2

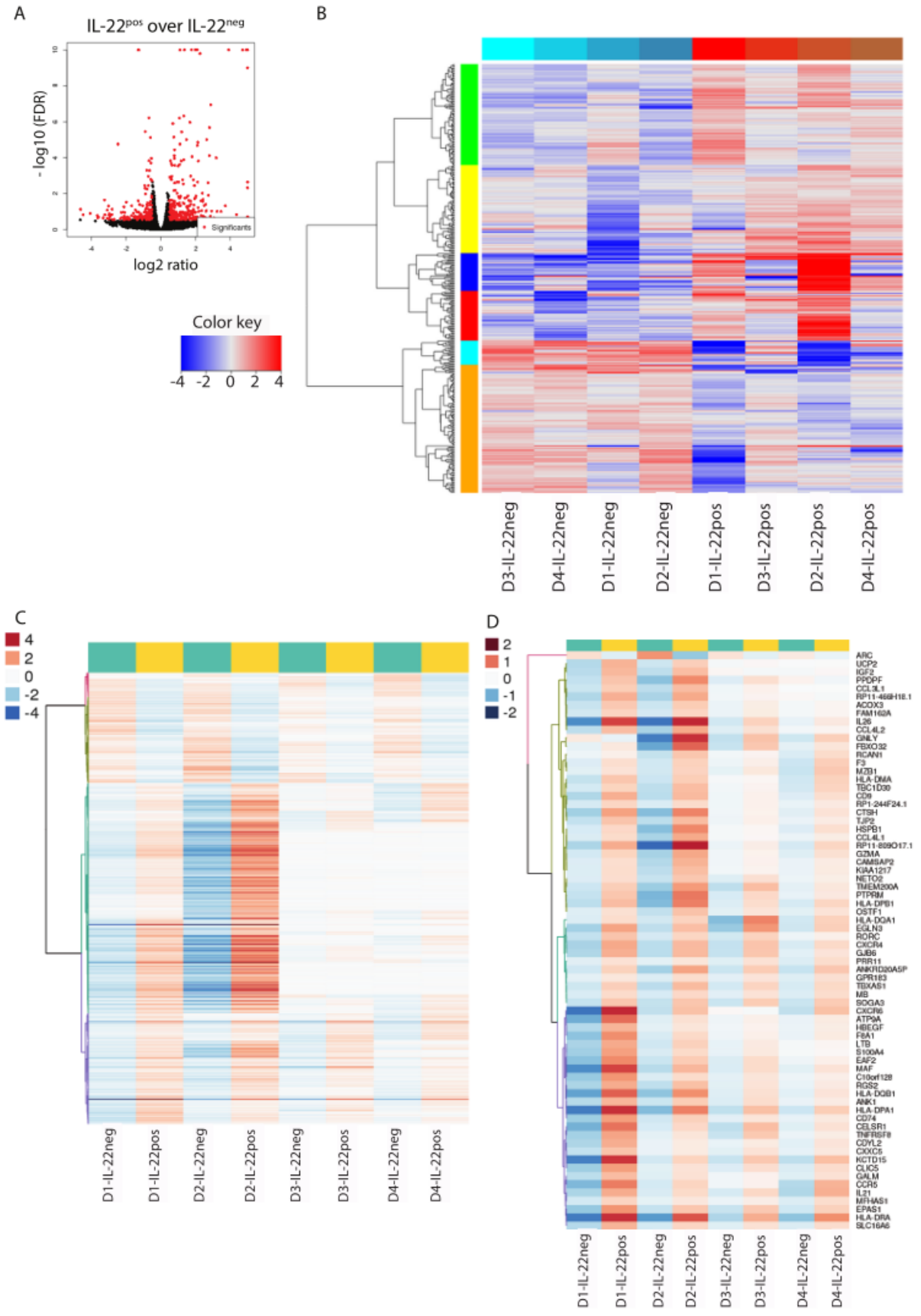
A



B



Supplementary figure 3



Supplementary figure 4

METHODS

Study group

The study has been reviewed and approved by the ethics committees of the Canton of Graubünden. Non-inflamed tonsils from patients undergoing tonsillectomy were obtained from the hospitals of Davos and Chur, Switzerland.

Purification of cells

Tonsil mononuclear cells (TMCs) were isolated from freshly removed palatine tonsils as previously described [228]. T cells were isolated from TMC`s with the use of human CD3 MicroBeads (Milteneyi Biotec, 130-050-101, Bergisch Gladbach, Germany) according to manufacturer`s protocol.

Intracellular cytokine staining

Thawed tonsil mononuclear cells that rested for 2h at 37^{o}C on the Tube Rotator (MACSmix Miltenyi Biotec, 130-090-753, Bergisch Gladbach, Germany) were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, P-8139, Buchs, Switzerland), 1 µg/ml Ionomycin (Sigma-Aldrich, I-0634, Buchs Switzerland) and 1 µg/ml of brefeldin A (Sigma-Aldrich, B-7651, Buchs, Switzerland) for 4 h in the 24-well plate at a concentration of 2x10^{6}/ml in cRPMI medium. After stimulation cells were washed in PBS and stained for 30 min at 4^{o}C with fixable viability dye eFluor 780 (eBioscience, 65-0865-14, Vienna, Austria) followed by staining with anti-human CD3 V500 (BD, 561416, Allschwil, Switzerland) and anti-human CD4 PC5.5 (Beckman Coulter, B16491, Krefeld, Germany). Next, cells were fixed and permeabilized with fixation/permeabilization solution for 20 min at 4^{o}C (BD Cytofix/Cytoperm, Fixation/Permeabilization solution kit, 554714, Allschwil, Switzerland). Later, cells were stained with anti-human IL-22 PE (eBioscience, 12-7229-42, Vienna, Austria), anti-human IL-17A Pacific Blue (Biolegend, 512312, London, UK), anti-human IL-4 AF488 (Biolegend, 500710, London,

UK), anti-human IL-13 APC (Biolegend, 501907, London, UK), anti-human IL-10 PE-CF594 (BD, 562400, Allschwill, Switzerland), anti-human IFN-γ AF700 (Biolegend, 506516, Londok UK) antibodies diluted in 100 µl of BD Perm/Wash buffer (BD, 554714, Allschwil, Switzerland) and incubated for 30 min at 4^{o}C.

Flow cytometry analysis

Cells were acquired with Galios Flow Cytometer (Beckman Coulter, Krefeld, Germany) and data was analysed with Kaluza Flow Cytometry Analysis Software ((Beckman Coulter, Krefeld, Germany). Co-production of cytokines by cytokine-producing T cells was analysed using TREE function of KALUZA software, which allows to calculate all possible combinations of coproduction of 5 cytokines by specific cytokine-producing cells. In this analysis, 100% of cells are considered as all cells, which are able to produce specific cytokine

Development of anti-IL-22 secretion assay

Purified anti-human CD45 antibody (eBioscience, 7014-0459-M001, Vienna, Austria) was conjugated with anti-human/mouse IL-22 purified antibody (eBioscience, 7013-7222-U500, Vienna, Austria) with the Pierce Controlled Protein-Protein Crosslinking Kit (ThermoFisher Scientific, 23456, Zug, Switzerland) according to manufacturer`s protocol. Formed in this way antibodies conjugate was used as anti-IL-22 catch reagent. IL-22 bound to the anti-IL-22 catch reagent was detected with anti-human IL-22 PE (eBioscience, 12-7229, Vienna, Austria). For the validation of anti-IL-22 catch reagent formation goat anti-rat IgG antibody was used (AF488 conjugated, Invitrogen, A11006, Zug, Switzerland)

Cell culture

Tonsil mononuclear cells have been cultured in the presence of 20 µg/ml TLR3 ligand Poly I:C (Sigma-Aldrich), IL-7 (40 ng/ml), and IL-23 (10 ng/ml)

in complete cRPMI medium (Gibco Cat. No. 21875-034, Paisley, Scotland) supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-Mercaptoethanol (all from Gibco). T cells isolated from tonsil mononuclear cells have been cultured with the mentioned above condition. IL-22-positive and IL-22-negative T cells that have been sorted with IL-22-secretion assay, were expanded in the cRPMI medium in the presence of 1 nM IL-2 as a growth factor. After 2 days of culture as well as after 20 days in culture (13 days plus 7 days after second re-stimulation) culture supernatants were collected and cytokines measured with Bio-Plex Pro Human Th17 Cytokine Assay (Bio-Rad, 171AA001M, Cressier, Switzeraland).

RNA isolation and sequencingAnalysis of RNA-seq data

Total RNA was isolated using the RNeasy Micro kit (Qiagen, 74034, Hombrechtikon, Switzerland) following the manufacturer's protocol. For validation of IL-22 secreiton assay mRNA from IL-22-positive and IL-22-negative was reverse transcribed with Revert Aid RT Kit (Thermo Scientific, K1691, Zug, Switzerland) according to manufacturer`s instructions. Real-time quantitative PCR was performed on a 7900HT Fast Real-Time PCR system (Thermo Scientific, 4329001, Zug, Switzerland) using Maxima Sybr Green/Rox qPCR Master Mix (Thermo Scientific, K0223, Zug, Switzerland) protocol with primers, IL-22 reverse primer: 5`TAG CAG CGC TCA CTC ATA CTG 3`, forward: 5`TTC ATG CTC GCCT AAG GAG GC 3`, IL-17a reverse primer: 5`CGG ACT GTG ATG TGC AAC CT3`, forward: 5` TCC TCA TTG CGG TGG AGA TT3`.

Next generation sequencing: RNA quality was assessed by Agilent 2100 Bioanalyzer, and the RNA integrity number was >7.0. The Library preparation for RNA-Seq was performed using the NuGEN Ovation Single Cell RNA-Seq System (NuGEN, San Carlos, CA) starting from 10 ng of total RNA. Libraries

were sequenced using HiSeq2500 from Illumina (single end; 1×100 bp). Sequence images were transformed with Illumina software BaseCaller to bcl files, which were demultiplexed to fastq files with CASAVA v1.8.2 (Illumina). Quality check was done via fastqc (v. 0.10.0, Babraham Institute, Cambridge, UK).

Raw sequencing reads were mapped to the Homo sapiens genome (GRCh37 build) using RSEM (v1.2.12) [PMID:21816040] implementation of Bowtie (v 1.0.0) [PMID:19261174] alignment program with the Ensemble annotation (v 75). Gene and isoform level abundances were quantified as RPKM values. Clustering analyses were performed using the 'ward.D2' clustering algorithm [331] implemented in the 'hclust' function of the R stats package. Heatmap plots were performed with the function 'heatmap.2' implemented in the gplots R package [https://cran.rproject.org/web/packages/gplots/index.html].

Differential expression analysis between the two conditions was performed using edgeR R bioconductor package [PMID:19910308]. Genes present in less than 75% in both condition were removed. Q-values were calculated using the Benjamini–Hochberg [332]and genes with a q-value < 0.05 and an absolute value of log 2 (fold change) > 1 were keep for further analyses.

Gene Ontology (GO) term enrichment analysis was performed using GOseq bioconductor package [PMID: 20132535] using the Wallenius approximation. In parallel, Pathway analysis was performed with MetaCore (version 6.23) from Thomson Reuters.

6.3 A novel, dual cytokine-secretion assay for the purification of human Th22 cells that do not co-produce IL-17A

Wawrzyniak Marcin[a], Ochsner Urs[a], Wirz Oliver [a], Wawrzyniak Paulina[a,b], van de Veen Willem[a], Akdis Cezmi A.[a,b], Akdis Mübeccel[a]

[a] Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland.
[b]CK-CARE AG - Christine Kühne – Center for Allergy Research and Education, Davos, Switzerland

Corresponding author:

Akdis Mübeccel,

Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Obere Strasse 22, Tel.: +41 81 410 08 48, akdism@siaf.uzh.ch

Highlights

- The combination of two cytokine-secretion assays allows the sorting of viable
  IL-22-producing cells as well as viable IL-17A-producing cells.
- For the first time, viable human IL-22-producing Th22 cells that do not co-produce IL-17A were purified.
- The combination of anti-IL-17A and anti-IL-22 secretion assays is comparable to intracellular staining in the demonstration of Th22 and Th17 cells

Keywords

Interleukin-22, Interleukin-17A, human Th22 cells, human Th17 cells, cytokine secretion assay, FACS (fluorescence-activated cell sorting)

ABSTRACT

Background

Interleukin-22 is produced by certain T helper cells subsets (Th17, Th22) and at lower levels by γδT, NKT and innate lymphoid cells. Th22 cells are unique immune cells that regulate tissue responses by IL-22 production. The exact discrimination between Th17 cells that co-produce IL-22 and single IL-22-producing Th22 cells has not been possible until the present study. Isolation of pure Th22 cells without co-expression of cytokines of other T cell subsets is essential to better understand their function in humans. The aim of the present study is the isolation and characterization of viable, human IL-22-producing CD4+ T cells that do not produce IL-17A.

Methods

Isolation of viable Th22 cells was performed with the combination of two cytokine secretion assays detecting IL-17A- and IL-22-producing cells in a single purification step.

Results

The newly developed cytokine secretion assay consists of anti-IL-22 and anti-IL-17A catch antibodies, which via biotin-streptavidin interaction are bound to the biotinylated surface of the target cell, and anti-IL-22 and IL-17A detection antibody labelled with a fluorescent dye, which detects cytokines bound to these catch antibodies. A unique population of human Th22 cells, which do not produce IL-17A, was sorted and cytokine expression pattern was confirmed by quantitative PCR analysis and ELISA. The presented technique allows the detection and isolation of pure human Th22 cells.

Conclusions

This technique may allow the purification of any single cytokine-producing cell subset, and the combination of several different cytokine secretion assays can be used to purify and characterize novel and unique cell subsets.

INTRODUCTION

Adaptive immune responses mediated by CD4+ T cells are necessary to eliminate viral, fungal or bacterial infections. They activate other immune cells by releasing T cell cytokines, assist B cells to produce antibodies, and regulate the immune system and tissue cells. CD4+ T cells can be characterized according to their cytokine production patterns [333]. New subsets of T helper cells were described during the last decade, including Th17 and Th22 cells [138, 146, 147]. There is still an ongoing debate and no clear distinction between Th17 and Th22 cells in humans, because the main cytokine of Th22 cells, IL-22, can be also produced by Th17 cells. Most of the confusion in the assigning IL-22 as Th17 rather than Th22 cytokine comes from discrepancy between mouse and human data. Among all mouse T helper subsets, Th17 cells are the major source of IL-22 and IL-22 is named as Th17 cytokine [141-143]. In contrast, IL-22 production by human cells does not correlate with either RORγt or IL-17A [139] and only 10-18% of IL-22-producing T cells in blood co-express IL-17A [138].

IL-22 together with IL-10, IL-19, IL-20, IL-24, IL-26, IL-28A, IL-28B, IL-29 is a member of the IL-10 cytokine family [121]. Cells of both, the innate and adaptive arms of the immune system, including αβ and γδ T cells, NKT and innate lymphoid cells, can produce IL-22. IL-22 was initially described as a cytokine produced by Th1 cells [126]. Although, IL-22 was co-produced by Th17 cells, it was later demonstrated that a fraction of human IL-22-producing T cells do not co-produce IL-17A, IL-4 or IFN-γ, which led to definition of new subset of T helper cells, named Th22 cells [138, 146, 147]. The main reason why IL-22 is considered a Th17 cytokine is that in mouse models, Th17 cells are the major source of IL-22. To date, characterization of human Th22 cells is mainly performed with *in vitro* manipulated T cell clones or cells expressing a combined chemokine receptor pattern, such as $CCR6^{+}CCR4^{+}CXCR3^{-}CCR10^{+}$

[138, 146]. However, these cells have not been shown to be proven representatives of Th22 cells as CCR10+CCR6+CCR4+ skin homing T cells are only enriched for T cells that produce IL-22 and most of them still co-produce other cytokines.

IL-22 binds to the IL-22 receptor (IL-22R), which consists of two subunits: IL-10R2 and IL-22R1 [119, 120]. IL-22 is a unique cytokine that is produced by immune cells and acts solely on non-immune tissue cells [334]. In general, the effects of IL-22 on epithelial cells in gut, skin and lungs can be divided into five main categories. Interleukin-22: 1) increases innate defense mechanisms against microbes via the induction of antibacterial protein production by epithelial cells [318, 335-337], 2) influences terminal differentiation of keratinocytes, 3) enhances the production of chemokines by epithelial cells [337], 4) and enhances migratory ability of epithelial cells [318, 338], and 5) shows a self-perpetuating action by upreguation of STAT3 and IL-20 [317, 339].

There is no consensus on the functions of single- and IL-17A/IL-22-co-producing cells so far. Uncontrolled IL-22-production can lead to pathogenesis, as demonstrated in psoriasis [171, 317], atopic dermatitis [340], rheumatoid arthritis [341], and *Toxoplasma gondii* infection [342]. One of the factors that may determinate the pro-inflammatory versus protective outcome of IL-22 action is the presence of IL-17A. For example, in the bleomycin induced acute airway inflammation model in mice, where Th17 cells co-produce IL-17A and IL-22, the disease is ameliorated in anti-IL-22 treated WT mice or IL-22-/- mice. Additionally, IL17-A-/- knockout mice are protected from airway inflammation, suggesting that IL-17A synergizes with IL-22 in this model [324]. These studies explain why it is important to understand the mechanisms of regulation of the production of both cytokines and the effects of single cytokine-producing versus IL-17A- and IL-22-co-producing cells. The present study describes combined IL-17A/IL-22 secretion assays allowing to purify and culture single IL-22-producing-Th22 cells, IL-17A-producing-Th17 cells and IL-22, IL-17A-co-

producing Th17/Th22 cells. This method may improve our understanding of the physiological and pathogenic functions of cells co-producing cytokines or producing them individually in various diseases, and can be used to identify other cell subsets co-expressing major cytokines.

METHODS

CD4+ T cell isolation

Peripheral blood mononuclear cells (PBMC`s) were isolated from buffy coats and whole blood using density gradient centrifugation (Biochrom, L6155, Berlin, Germany). CD4+ T cells were isolated using an anti-human CD4+ T cells isolation kit (Miltenyi Biotec, 130-096-533, Bergisch Gladbach, Germany) and cultured overnight at 37^{o}C for further experiments in cRPMI 1640 medium (Gibco Cat. No. 21875-034, Paisley, Scotland) supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-Mercaptoethanol (all from Gibco)

Biotinylation of cell surface proteins

Cells surface proteins were biotinylated using Pierce Premium Grade Sulfo-NHS-LC-Biotin (Thermo Scientific, PG82075, Zug, Switzerland) according to the manufacturer`s instructions. Briefly, 10x10^{6} cells were washed with PBS pH 8, then stained with 1 mg/ml of Sulfo-NHS-LC-Biotin and incubated for 30 min at room temperature. The dose of Sulfo-NHS-LC-Biotin was optimized in preliminary experiments (data not shown). After incubation, cells were washed 3 times with PBS pH 8 supplemented with 100 mM glycine. Final washing of cells was performed with PBS supplemented with 0.5% bovine serum albumin (BSA) (Sigma, A9418-500G, Buchs, Switzerland) and 2 mM EDTA (Suppl. Figure 3).

Validation for biotinylation of cell surface proteins

Biotinylated and non-biotinylated cells were stained for 5 min on ice, with phycoerythrin (PE)- labelled streptavidin at a concentration of 6.25 µg/ml (BD Pharmingen, 554061, Allschwil, Switzerland). Both, biotinylated and

non-biotinylated cells were then co-stained with 5 µl (0.25µg) of anti-human CD3 BV421 (Biolegend, 300434, London, UK) antibodies and 5µl (0.25µg) and 7AAD viability dye (BD Pharmingen, 559925, Allschwil, Switzerland) for 10 min on ice. Flow cytometric analysis was performed with BD FACS ARIA II (BD, Allschwil, Switzerland).

Proliferation analysis of biotinylated cells

Equal amounts of PBMCs were biotinlyated or left unbiotinylated. All cells were then labelled with cell trace violet dye (CTV; Life Technologies, C34557, Zug, Switzerland). For this, CTV was diluted in pre-warmed PBS (37°C) to a working concentration of 1.25 µM. Cell pellets were resuspended with CTV dye solution at a concentration of $1x10^6$ cells/ml and incubated for 20 min at 37°C on a tube rotator to assure uniform labelling. Following this, five times the original staining volume of pre-warmed medium was added and incubated for 5 min to remove nonspecific CTV dye in the solution. Next, cells were washed and resuspended in cRPMI medium. Biotinylated and non-biotinylated cells were then cultured for 6 days, stimulated with a mixture of anti-CD3, CD2, CD28 mAbs (0.5 ug/ml) or unstimulated. On day 6, cells were stained with Fixable Viability Dye eFluor 780 (eBioscience, 65-0865-14, Vienna, Austria) and anti-human CD3 BV510 antibody (Biolegend, 300448, London, UK).

Streptavidin labelling of anti-cytokine catch antibodies

Purified anti-human IL-22 (eBioscience, 12-7229-42, London, UK) and anti-human IL-17A (eBioscience, 14-7178-85, London, UK) antibodies were labelled with streptavidin using Lightning-Link Streptavidin Conjugation kit (Innova Biosciences, 708-0010, Cambridge, UK) according to the manufacturer`s instructions. Total amount of 100 µg of both anti-cytokine antibodies were used for streptavidin labelling.

Titration of anti-cytokine catch antibodies

Streptavidin labelled, anti-cytokine catch antibodies were added to PBMC`s at a concentration of $10x10^6$ cells in different concentrations, starting from 10 µg/ml, 5 µg/ml, 2.5 µg/ml to 1.25 µg/ml and incubated for 20 min on ice. Next, goat anti-rat IgG (AF488 conjugated, Invitrogen, A11006, Zug, Switzerland) and goat anti-mouse IgG (AF633 conjugated, Invitrogen, 31575, Zug, Switzerland) antibodies were used in 20 µg/ml concentration to detect rat anti-human IL-22 and mouse anti-human IL-17A streptavidin-labelled catch antibodies bound to biotinylated cells surface, respectively. Staining was performed on ice for 20 min. Additionally, cells were stained with anti-CD3 BV421 mAb and 7AAD for 10 min.

Intracellular cytokine staining

After resting overnight, CD3+CD4+ T cells were stimulated in 24 well plates at a concentration of $2x10^6$/ml in cRPMI medium for 4 hours with 25 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, P-8139, Buchs, Switzerland) and 1 µg/ml Ionomycin (Sigma-Aldrich, I-0634, Buchs Switzerland). Following this, 1 µg/ml of brefeldin A (Sigma-Aldrich, B-7651, Buchs, Switzerland) was added for additional 2 hours. After stimulation, cells were washed in PBS and stained with fixable viability dye eFluor 780 for 30 min at 4^oC (eBioscience, 65-0865-14, Vienna, Austria) followed by staining with 5 µl (0.25 µg) of anti-human CD3 (BV421, Biolegend, 300434, London, UK) and 5 µl (1 µg) of anti-human CD4 for 15 min at 4^oC (PE-Cy7, Biolegend, 400325, London, UK). Before intracellular staining, cells were fixed and permeabilized with fixation/permeabilization solution for 20 min at 4^oC (BD Cytofix/Cytoperm Fixation/Permeabilization solution kit, 554714, Allschwil, Switzerland). Next, 5 µl (0.25 µg) of anti-human IL-17A AF488 (eBioscience, 53-7179, Vienna, Austria) and 5 µl (0.06 µg) of anti-human IL-22 PE (eBioscience, 12-7229, Vienna, Austria) antibodies diluted in 100 µl of BD

Perm/Wash buffer (BD, 554714, Allschwil, Switzerland) were used for intracellular cytokine staining for 30 min at 4^{o}C.

Sorting of IL-17A- and IL-22-secreting CD4+ T cells by a dual cytokine secretion assay

After biotinylation as described above, cells were stained with fixable viability dye eFluor 780 for 30 min and anti-human CD3 (BV421, Biolegend, 300434, London, UK) and anti-human CD4 (PE-Cy7, Biolegend, 400325, London, UK) antibodies for 15 min on ice. Next, cells were stained with streptavidinylated anti-IL-22 and anti-IL-17A catch antibodies at a concentration of 10µg/ml for 20 min on ice. Cells that were not stained with streptavidinylated antibodies served as negative controls. For the cytokine secretion, cells were stimulated with 25 ng/ml PMA, 1 µg/ml Ionomycin in cRPMI medium at the concentration of 1x10^{6} and incubated for 4 hours on the Tube Rotator (MACSmix Milteneyi Biotec, 130-090-753, Bergisch Gladbach, Germany). After incubation, cells were stained with anti-human IL-17A AF488 and anti-human IL-22 detection antibodies for 20 min on ice. It will be good to note here that the same antibodies were used for intracellular staining as well. Four different populations of cells were sorted into PBS based on gates set up with "no-catch antibody reagent" controls: 1) IL-17A-producing IL-22-non-producing Th17 cells, 2) IL-17A-non-producing and IL-22-producing Th22 cells, 3) both IL-17A- and IL-22-producing Th17/Th22 cells and 4) cells producing neither IL-22, nor IL-17A.

RT-PCR detection of cytokines in sorted cells

Sorted cells were lysed in RLT Plus RNA lysis buffer (Qiagen, 74034, Hombrechtikon, Switzerland). Total RNA was isolated using RNeasy Plus Micro kit (Qiagen, 74034, Hombrechtikon, Switzerland). mRNA was reverse transcribed with Revert Aid RT Kit (Thermo Scientific, K1691, Zug, Switzerland) according to manufacturer`s instructions. Real-time quantitative

PCR was performed on a 7900HT Fast Real-Time PCR system (Thermo Scientific, 4329001, Zug, Switzerland) using Maxima Sybr Green/Rox qPCR Master Mix (Thermo Scientific, K0223, Zug, Switzerland) protocol with primers, IL-22 reverse primer: 5`TAG CAG CGC TCA CTC ATA CTG 3`, forward: 5`TTC ATG CTC GCCT AAG GAG GC 3`, IL-17a reverse primer: 5`CGG ACT GTG ATG TGC AAC CT3`, forward: 5` TCC TCA TTG CGG TGG AGA TT3`. Primers sequences used to assess phenotypic differences between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} cells are stated in supplementary table 1. Results were normalized to EF1α (eukaryotic translation elongation factor 1 alpha) expression for each sample. Gene expression levels, shown in arbitrary units, were calculated by subtracting EF1 α cycle threshold (Ct) from the gene Ct to get ΔCt value. Arbitrary units for each sample = 10 000 x ($2^{-\Delta Ct}$).

Culture of sorted Th22 and Th17 cells

Purified Th22 and Th17 cells were expanded in the presence of 1 nM IL-2 as a growth factor. After 10 days of expansion, T cells were re-stimulated with a combination of anti-CD2, anti-CD3 and anti-CD28 (0.5 ug/ml) monoclonal antibodies in cRPMI medium. Supernatants were harvested after 48 h and IL-22 and IL-17A were measured with human Th17 magnetic bead panel kit (HTH17MAG-14K, Milliplex, Schaffenhausen, Switzerland).

RESULTS

Validation of dual cytokine-secretion assay

The complete cytokine secretion assay consists of streptavidin-labelled anti-cytokine catch antibodies, which are bound to the biotinylated surface of the cells, and fluorescently labelled cytokine detection antibodies (Suppl. Figure 1). Biotin labelling of cell surface proteins is the first step of the novel dual cytokine-secretion assay. To prove that this step was done correctly, the detection of biotinylated cell surface using a PE-labelled streptavidin was performed. As demonstrated in Figure 1A, only biotinylated cells were stained with streptavidin-PE, as compared to non-biotinylated cells, with approximately 98 % of viable, CD3+ T cells stained with streptavidin-PE, demonstrating that biotinylation of cell surface proteins was successful (Figure 1A, B). The reagent (Sulfo-NHS-LC-Biotin) that was used to biotinylate the cells is membrane impermeable, water-soluble and biotinylates only primary amines present on the surface of the cells. In accordance with that fact, 93.14 ± 1.52 % of biotinylated CD3+ T cells were viable, as compared to 96.17 ± 1.52 % of viable non-biotinylated cells, demonstrating that biotinylation had no effect on cell viability (Figure 1C).

Next, to investigate, if biotinylation activates the cells and induces their proliferation, peripheral blood mononuclear cells (PBMC`s) were stimulated with an anti-CD2/CD3/CD28 mixture of mAbs and stained with a cell division tracking (CTV) reagent. On day 6, 94.0 ± 6.6 % of biotinylated and 92.7 ± 6.3 % of non-biotinylated CD3+ T cells proliferated in response to T cell specific stimuli. Furthermore, spontaneous proliferation of non-stimulated, biotinylated cells was not observed (Figure 1D), (Suppl. Figure 2). These data prove that

surface biotinylation of T cells influence neither their viability nor their ability to proliferate.

After biotinylation, cells were ready for the next step in the cytokine secretion assay procedure: Staining with anti-cytokine catch antibodies. Here, we took the advantage of the fact that streptavidin has a very high affinity for biotin ($K_d \approx 10^{-14}$) [343], and we labelled purified anti-cytokine catch antibodies with streptavidin and used them to bind to biotin on the surface of the cells. To find the optimal concentration of streptavidin-labelled catch antibodies, which cover the whole cell surface, a 10, 5, 2.5, to 1.25 µg/ml titration of both antibodies was performed (Figure 2A). To detect these catch antibodies (Ab`s) on the surface of the cells, we took the advantage that anti-human IL-22 antibody (Ab) was of rat origin and anti-human IL-17A Ab was of mouse origin. Therefore, we used goat anti-rat IgG AF488 Ab to detect anti-IL-22 catch antibody, and goat anti-mouse AF633 Ab to detect anti-IL-17A catch antibody on the surface of cells (Suppl. Figure 3). As shown in Figure 2B, concentration-dependent labelling of the cells was observed, with 1.25 µg/ml and 2.5 µg/ml of antibodies not being sufficient to cover the whole surface of cells. Only Ab`s at concentration of 5 and 10 µg/ml enabled a complete staining of the cell population. As shown on Figure 2B, the percentage of cells labelled with both catch antibodies in case of 10 µg/ml concentration reached 97,8 ± 0.75%, we decided to use for further assays anti-cytokine catch antibodies at a concentration of 10 µg/ml. Dual secretion assay was capable of catching IL-22 and IL-17A cytokines produced by the cells. Secreted cytokines were bound to catch antibodies on the surface of cells and later detected with fluorescently labelled detection antibodies.

IL-22- and IL-17A-secretion assays recognize cytokine-producing CD3+CD4+ T cells in similar quantities compared to intracellular cytokine staining

To analyse intracellular cytokine production, cells were gated on viable CD3+CD4+ T cells and IL-22 and IL-17A production was determined (Figure 3A). As presented in Figure 3B, the percentage of IL-22^{pos} cells detected with secretion assay positively correlated with the percentage of IL-22^{pos} cells identified with intracellular staining. More interestingly, similar correlation, was found for Th22 cells, which produced IL-22 and didn`t produce IL-17A. In addition, positive correlation between secretion assay and intracellular staining was observed for IL-17A-producing cells: IL-17A^{pos}IL-22^{pos} cells and IL-17A^{pos}IL-22^{neg} cells. We were able to detect cell populations as low as 0.05% and 0.06% of IL-22+ and IL-17A+ T helper cells with the dual secretion assay (Suppl. Figure 4), suggesting that this secretion assay is as sensitive as intracellular cytokine staining.

Purified Th22 cells are characterized by high expression of IL22, but not IL17a mRNA

In the next step, IL-17A-single positive, double IL-17A/IL-22-producing, IL-17A/IL-22-double negative and most importantly, IL-22-single positive cells were sorted (Suppl. Figure 4A). During sorting, cells that were not stained with anti-cytokine catch antibodies, but were stained with fluorescently labelled detection Ab`s, served as controls. Based on these controls, gates were set for better estimation of cytokine production by T cells. To avoid cytokine-non-producing cells non-specifically binding the cytokines produced by neighboring cells, the cells were suspended at a concentration of $1x10^6$/ml and incubated on a tube rotator to provide constant mixing of cells, thereby avoiding cell-cell contact. Although, the production of both cytokines varied between different donors, staining with secretion assays allowed sorting of 4 separate populations of cells. The percentage of IL-22^{pos}IL-17A^{neg} T cells was significantly higher than the percentage of IL-22^{pos}IL-17A^{pos} T cells ($p \leq 0.001$) suggesting that there are more Th22 cells than Th17/Th22 cells in peripheral blood (Figure 4A).

Subsequently, mRNA was isolated from sorted cells and the expression of IL22 and IL17A mRNA was quantified by RT-qPCR (Figure 4B). IL-22 expression was only detected in IL-22-single positive and IL-17A/IL-22-double positive cells, but not in IL-17A-single positive and IL-17A/IL-22-double negative cells. Similar results were obtained for IL-17A mRNA expression, with IL-17A detected only in IL-17A-single positive and IL-17A/IL-22-double positive cells. Interestingly, IL22 mRNA was significantly higher expressed in IL-22-single positive and double positive cells as compared to double negative cells. Moreover, analogous to this finding it was reported for IL17A mRNA expression, as IL17A was expressed significantly higher in IL-17A-single positive as well as in double positive cells when compared to double negative cells.

In addition, the expression of chemokine receptors, T helper cell-related transcription factors and cytokines were analyzed in IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells (Suppl. Figure 6). IL-22^{neg} IL-17^{pos} cells expressed higher mRNA levels of CCR6 and CCR4 chemokines receptors and RORC2 transcription factor compared to IL-22^{pos} IL-17^{neg} cells. There was no difference in mRNA expression of CCR10, CXCR3, CLA, T-bet, Gata-3, AHR, IFN-γ and IL-4 between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} T cells.

Furthermore, IL-22^{pos}IL-$17A^{neg}$ Th22 cells were purified and expanded in culture for 12 days. They were characterized by production of IL-22 and little or no production of IL-17A (Figure 4C). A similar finding was observed for IL-22^{neg}IL-$17A^{pos}$ Th17 cells, which did not produce IL-22, but produced IL-17A (Figure 4C). In conclusion, these results confirm that dual secretion assay detects accurately Th22 as well as Th17 cells, and sorted Th22 and Th17 cells show a stable phenotype over the course of 12 days in culture.

DISCUSSION

The first cytokine secretion assay was established in 1995 by Manz and colleagues to sort viable cells according to their secreted molecules, secreted to a cell-surface affinity matrix [344]. Since then, some commercially available secretion assays were developed and consist of anti-cytokine catch reagent (anti-CD45 Ab linked to an anti-cytokine Ab) and detection Ab binding to a different epitope of the cytokine of interest, which is labelled with a fluorescent dye. The main restriction of above-mentioned secretion assays is that limited number of fluorescently labelled detection antibodies exists and IL-22-secretion assay has not been developed yet.

For a long time, IL-22-producing cells were considered as a subset of Th17 cells [142]. This was mainly due to the fact that, there are only a few tools available to study human Th22 cells, such as intracellular cytokine staining, generation of T cell clones and cell sorting based on chemokine receptors [138, 146, 147]. In the present study, we show that a combination of dual anti-IL-22 and anti-IL-17A secretion assays, allow to sort viable IL-22-producing Th22 cells, that do not co-express IL-17A. This novel method expands the possibilities to further characterize human Th22 cells, because cells sorted with this secretion assay can be sterile handled and characterized, co-cultured with other cells or used in humanized mouse models.

One of the novel and important steps in this currently presented dual-secretion assay is the biotinylation of cell surface proteins. Our data show that biotinylation of cells did not affect cell viability and the ability of cells to proliferate without any reservation. Moreover, biotinylated cells do not

proliferate spontaneously. Together, these results suggest that, sorted, biotinylated cells can be cultured *in vitro.*

In the next step, streptavidin labelled anti-cytokine antibodies were used to catch cytokines produced by cells. At this step, it was possible to combine more than two anti-cytokine catch antibodies, as long as detection antibodies used in next step recognize different epitopes of the same cytokine and were labelled with different fluorochromes. In the present study, two catch antibodies were used, which allowed us to sort Th22 cell that do not express IL-17A and Th17 cells that do not express IL-22. After culture, the sorted cells kept their profile of the initial sorting strategy with Th22 cells producing IL-22 and low amounts of IL-17A, and Th17 cells producing IL-17A and not producing IL-22.

The measurement of transcription factors and chemokines provided further evidence on the purity and functional relevance of Th17 and Th22 cells. IL-22^{neg} IL-17^{pos} T cells expressed higher levels of CCR6 and CCR4 chemokine receptors compared to IL-22^{pos} IL-17^{neg} cells suggesting that previously reported pattern of chemokines receptors expression - CCR6^{+}CCR4^{+}CXCR3^{-} [138, 146], better describes Th17 rather than Th22 cells. As expected, IL-22^{neg} IL-17^{pos} expressed higher levels of RORC2 transcription factor compared to IL-22^{pos} IL-17^{neg} T cells. AhR, has been reported to be essential for IL-22 expression in Th17 cells [151, 158], γδ T cells [158] and human Th22 cells [138, 146] in some studies, whereas others have reported that AHR is not absolutely required for IL-22 expression [286]. Although it was expressed in relatively high levels in both cell subsets, AhR expression was not different between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} cells in the present study.

To further validate the cytokine profile and quality of purified cells, IL-17A and IL-22 production by T cells was compared between intracellular staining and the secretion assay. During intracellular staining, permeabilization and fixation of cells renders them useless for further functional experiments. In addition, protein transport from endoplasmic reticulum (ER) to Golgi apparatus

is blocked with brefeldin A during intracellular staining. This method gives only information about the ability of cells to produce cytokines, without revealing their extracellular secretion. In contrast, cell sorting based on their cytokine production with a secretion assay gives a capability to culture viable cells and study their biology in detail. The cells detected with a secretion assay are the cells, which secrete cytokines into the extracellular space resembling more closely the *in vivo* situation. For both presented methods, cells were stimulated with PMA and ionomycin, but in case of the secretion assay stimulation took place after surface staining, during incubation on tube rotator at 37^{o}C, and in case of intracellular staining, it took place before surface staining and was followed by the addition of brefeldin A. Nevertheless, the percentages of cells detected with the secretion assay significantly correlated with percentages of cells detected with intracellular staining, suggesting that secretion assay is as sensitive in the detection of cytokine-producing cells as intracellular cytokine staining.

In conclusion, for the first time, we were able to sort pure, human Th22 cells based on their IL-22 production, which are lacking IL-17A production. Sorted cells could be cultured at a later stage and their cytokine profile investigated. The combination of two secretion assays, as presented here, gives a possibility to sort different subsets of Th22 cells, co-producing other cytokines, such as IL-17A and investigate the interaction of these cells with other cells. Understanding the differences in the regulation of IL-22 may provide an insight into the pathogenic and physiological functions of this cytokine in various diseases. This methodology will enable even more than two cytokines to be analyzed at the same time. As soon as the pair of antibodies, which detect different epitopes of the same cytokine exist, and one of them is labelled with a fluorescent dye, multiple anti-cytokine secretion assays may be combined for more precise analyses of single or combined cytokine-secreting cells.

Acknowledgement

Work supported by Swiss National Science Foundation (grant nos. 320030-125249/1, 32-188226, and 320030-140772) and the European Commission`s Seventh Framework Programme (grant agreement no. 261357 [MEDALL]). First author would like to thank David Groeger for proof-reading the manuscript.

Author Contributions

Wawrzyniak Marcin - Conception and design of experiments, data acquisition, analysis and interpretation, drafting the article.

Ochsner Urs - Substantial contributions to acquisition, analysis and interpretation of data.

Oliver Wirz - Substantial contributions to acquisition, analysis and interpretation of data.

Wawrzyniak Paulina - Substantial contributions to acquisition, analysis and interpretation of data.

Van de Veen Willem - Revising article critically for the important intellectual content, final approval of the version to be published.

Akdis Cezmi A. - Revising the article critically for the important intellectual content, final approval of the version to be published.

Akdis Mübeccel - Design of experiments, revising the article critically for the important intellectual content, final approval of the version to be published.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Figure legends

Figure 1. Validation of cell surface biotinylation.

Biotinylated and non-biotinylated PBMCs were stained with streptavidin-PE to examine the efficiency of biotinylation process (A). In the gate of viable CD3+ T cells, 97.32 ± 4.52% of biotinylated CD3+T cells were stained with streptavidin - PE (B). The biotinylation of cells does not influence cells viability, as the percentages of viable CD3+T cells were similar among biotinylated and non-biotinylated cells (n=3) (C). Next, proliferation of biotinylated and non-biotinylated cells in response to anti-CD2, CD3, CD28 mAb stimulation was investigated. Similar percentages of biotinylated and non-biotinylated CD3+T cells responded to anti-CD2/CD3/CD28 stimulation on day 6 after starting the cultures as measured using the cell proliferation dye (CTV). Biotinylation of the cell surface did not induce spontaneous proliferation of biotinylated cells (n=4) (D). Two-way Anova test with multiple comparisons was applied (**** $p<0.0001$).

Figure 2. Titration of anti-cytokine catch antibodies binding to the cell surface.

Binding of streptavidin-labelled anti-cytokine catch antibodies to biotinylated cell surface was analysed, and the titration of the anti-cytokine catch antibodies was performed (A). Rat anti-human IL-22 and mouse anti-human IL-17A catch antibodies were detected with goat anti-rat AF488 and goat anti-mouse AF633 antibodies, respectively (B). Concentration-dependent labelling was observed, and optimal dose of 10 µg/ml of both catch antibodies was established (n=4). One-way Anova test with multiple comparisons was calculated (*$p<0.05$, ** $p<0.01$)

Figure 3. Comparison of cytokine production detected by secretion assays and intracellular staining.

The production of IL-17A and IL-22 was analysed using both, the secretion assay and intracellular while gating on staining viable, CD3+CD4+T cells (A). The percentage of CD4+IL-22+, CD4+IL-17A+ T cells detected in secretion assay correlated with results

obtained with intracellular staining (B). A strong correlation was observed for IL-17A^{neg}IL-22^{pos} Th22 cells, IL-17A^{pos}IL-22^{pos} Th17/Th22 cells and for IL-17A^{pos}IL-22^{neg} Th17 cells (B). Each dot represents a separate donor (n=7). Non parametric Spearman correlation coefficient r was calculated.

Figure 4. Sorting and characterization of IL-22-producing Th22 cells and IL-17A-producing Th17 cells.

CD3+CD4+T cells were stained with anti-IL-17A and IL-22 secretion assays and four different populations of cells were sorted (IL-22^{pos}IL-17A^{neg}, double positive, IL-17A^{pos} IL-22^{neg}, double negative), expression of IL17A and IL22 was measured with RT-PCR (B). IL22 expression was only detected in IL-22^{pos}IL-17A^{neg} and double positive cells. Whereas, expression of IL-17A was detected only in double positive and IL-22^{neg}IL-17A^{pos} T cells (n=5) (B). The percentage of IL-22-positive cells, which do not produce IL-17A varied from 0.074% to 0.72% of total CD3+CD4+ T cells (A) (n=7) and were significantly higher in number as compared to IL-17A/IL-22-double positive cells. No difference in number of CD4+IL-17A+ and CD4+IL-22+ cells was observed. Sorted IL-22^{pos}IL-17A^{neg} and IL-22^{neg}IL-17A^{pos} cells were cultured in cRPMI medium supplemented with IL-2 for 12 days and after the second round of T cells re-stimulation with anti-CD2/CD3/CD28 antibodies, production of IL-22 and IL-17A was measured by luminometric bead array. Th22 cells produced IL-22 with a very low amount of IL-17A, in contrast Th17 cells produced IL-17A with no-co production of IL-22 (C). One-way Anova test for repeated measures was applied (*$p<0.05$, ** $p<0.01$, ***$p<0.001$)

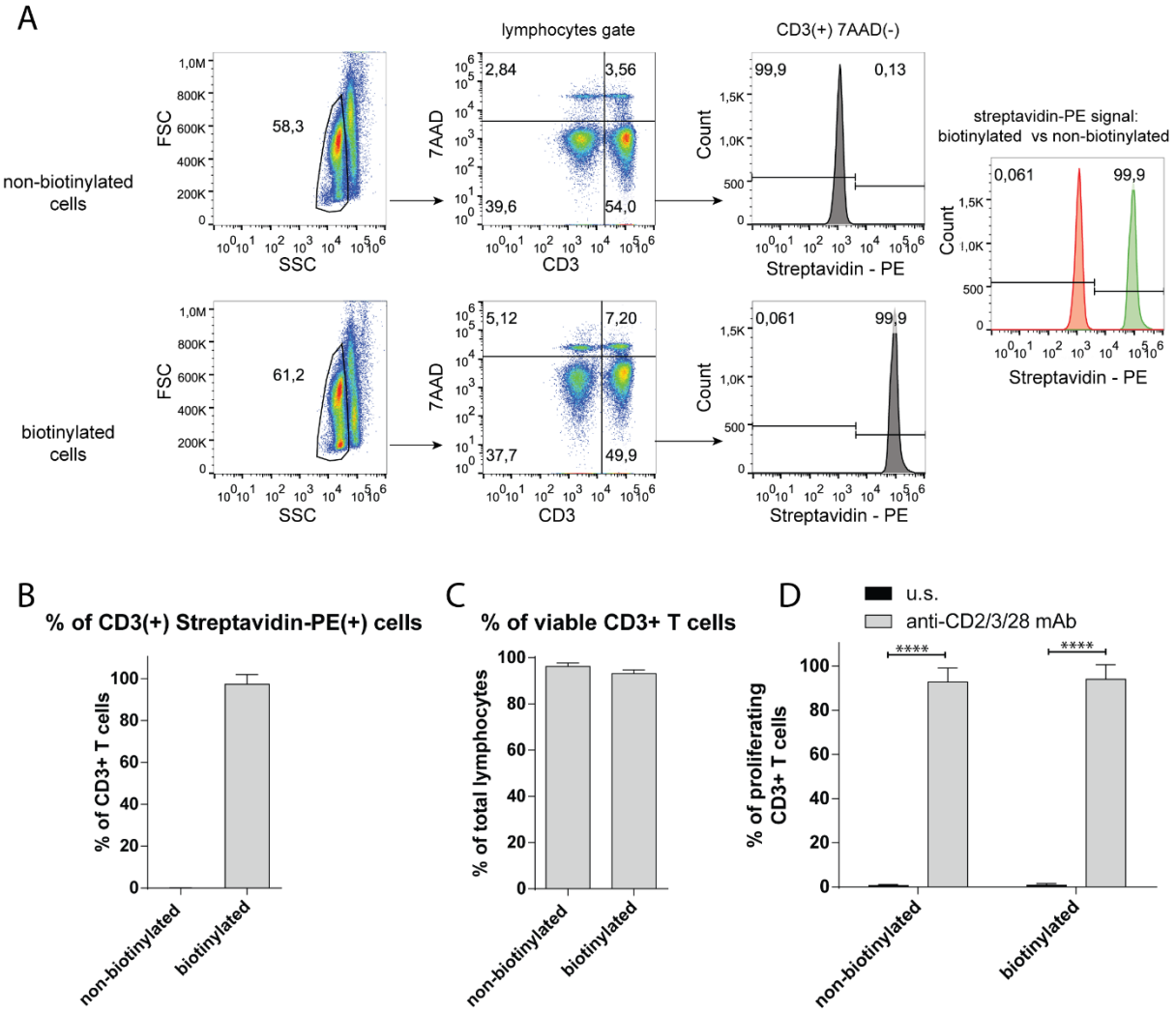

A
lymphocytes gate
CD3(+) 7AAD(-)
non-biotinylated cells
biotinylated cells
FSC
SSC
58,3
61,2
7AAD
CD3
2,84
3,56
39,6
54,0
5,12
7,20
37,7
49,9
Count
Streptavidin - PE
99,9
0,13
0,061
99,9
streptavidin-PE signal: biotinylated vs non-biotinylated
B
% of CD3(+) Streptavidin-PE(+) cells
% of CD3+ T cells
non-biotinylated
biotinylated
C
% of viable CD3+ T cells
% of total lymphocytes
D
u.s.
anti-CD2/3/28 mAb
% of proliferating CD3+ T cells
****

Figure 1

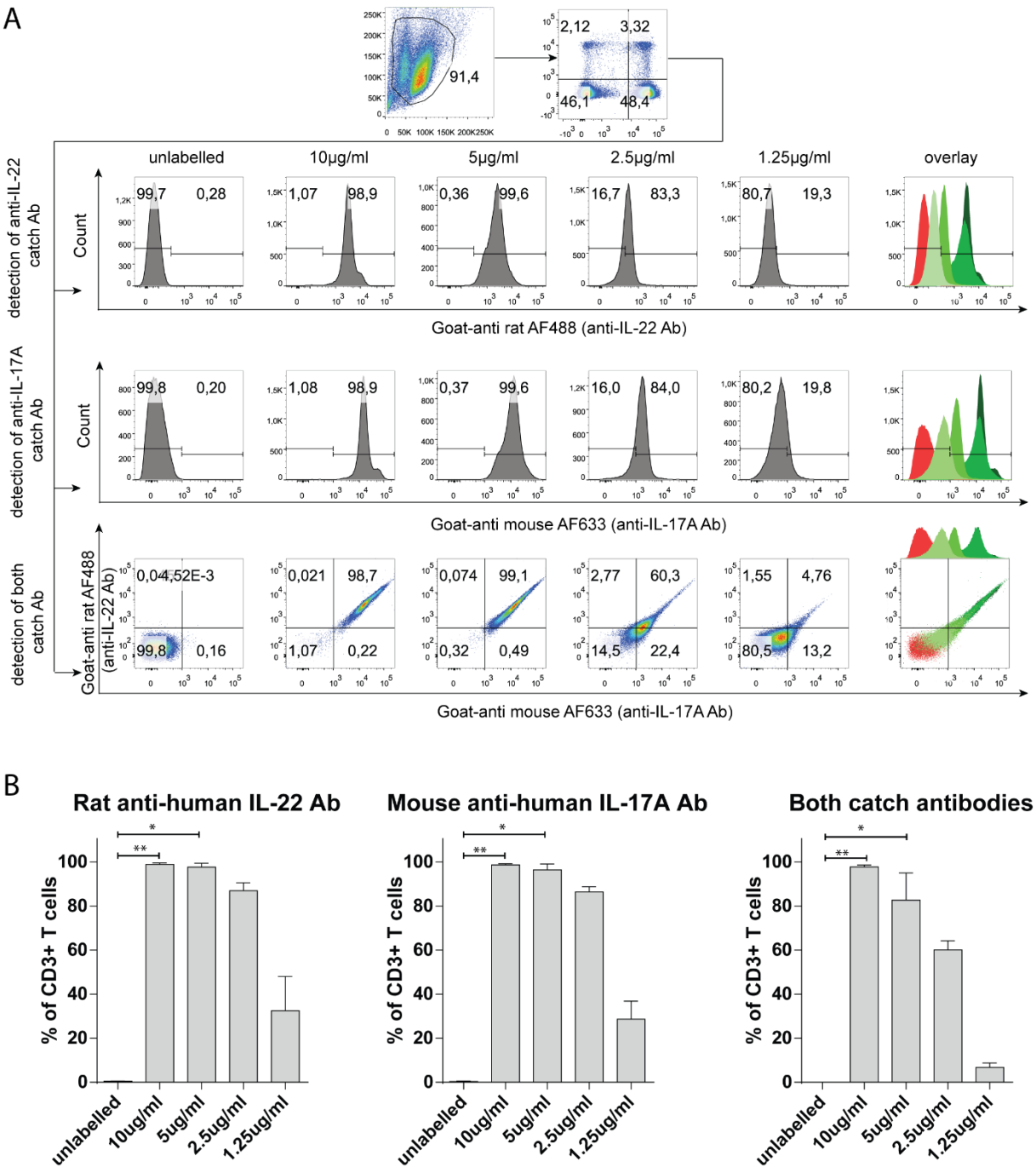

A
91,4
2,12
3,32
46,1
48,4
unlabelled
10µg/ml
5µg/ml
2.5µg/ml
1.25µg/ml
overlay
detection of anti-IL-22 catch Ab
Count
99,7
0,28
1,07
98,9
0,36
99,6
16,7
83,3
80,7
19,3
Goat-anti rat AF488 (anti-IL-22 Ab)
detection of anti-IL-17A catch Ab
99,8
0,20
1,08
98,9
0,37
99,6
16,0
84,0
80,2
19,8
Goat-anti mouse AF633 (anti-IL-17A Ab)
detection of both catch Ab
Goat-anti rat AF488 (anti-IL-22 Ab)
0,04
52E-3
0,021
98,7
0,074
99,1
2,77
60,3
1,55
4,76
99,8
0,16
1,07
0,22
0,32
0,49
14,5
22,4
80,5
13,2
Goat-anti mouse AF633 (anti-IL-17A Ab)
B
Rat anti-human IL-22 Ab
Mouse anti-human IL-17A Ab
Both catch antibodies
% of CD3+ T cells
unlabelled
10ug/ml
5ug/ml
2.5ug/ml
1.25ug/ml

Figure 2

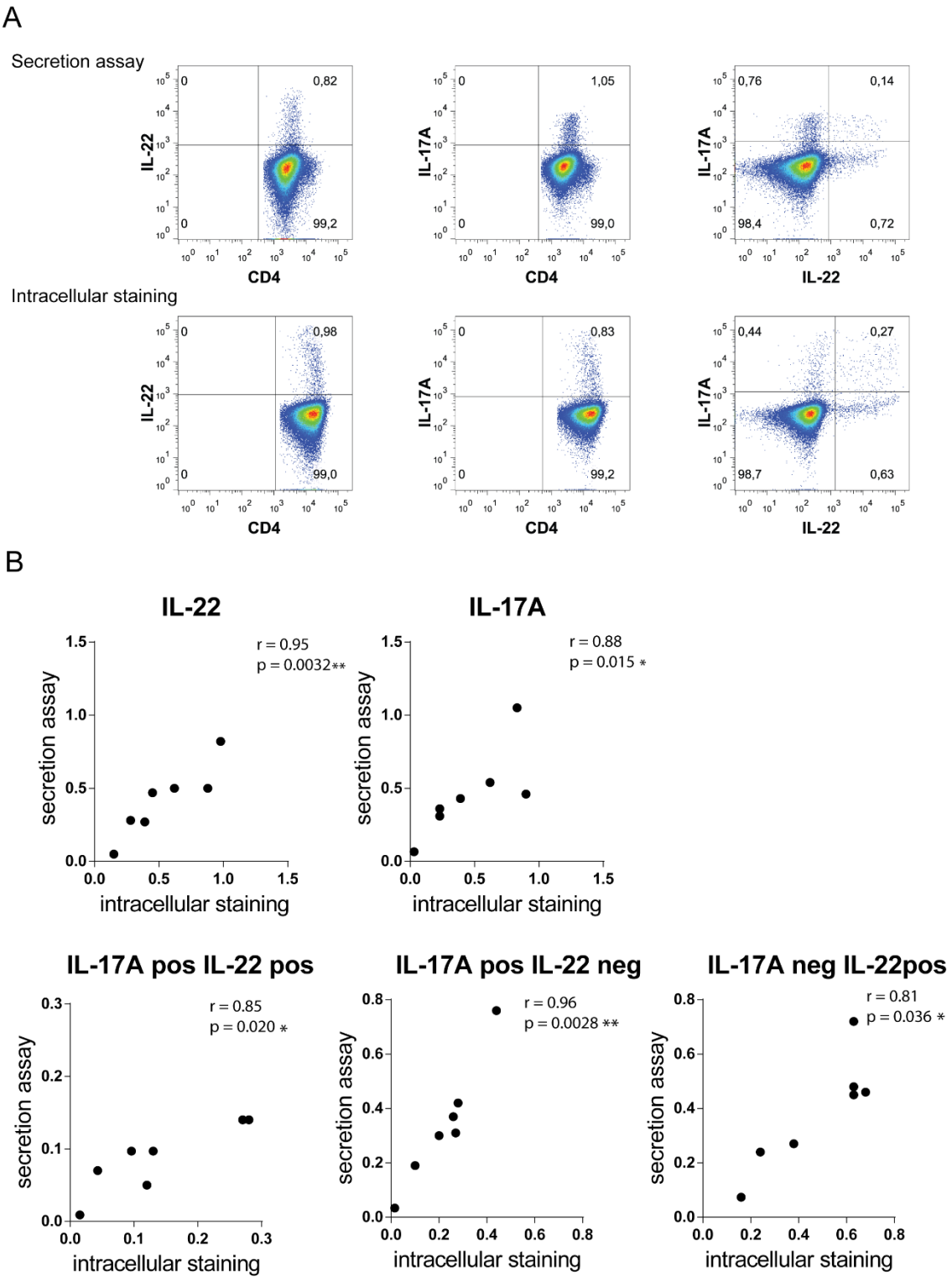


Figure 3.

Figure 3

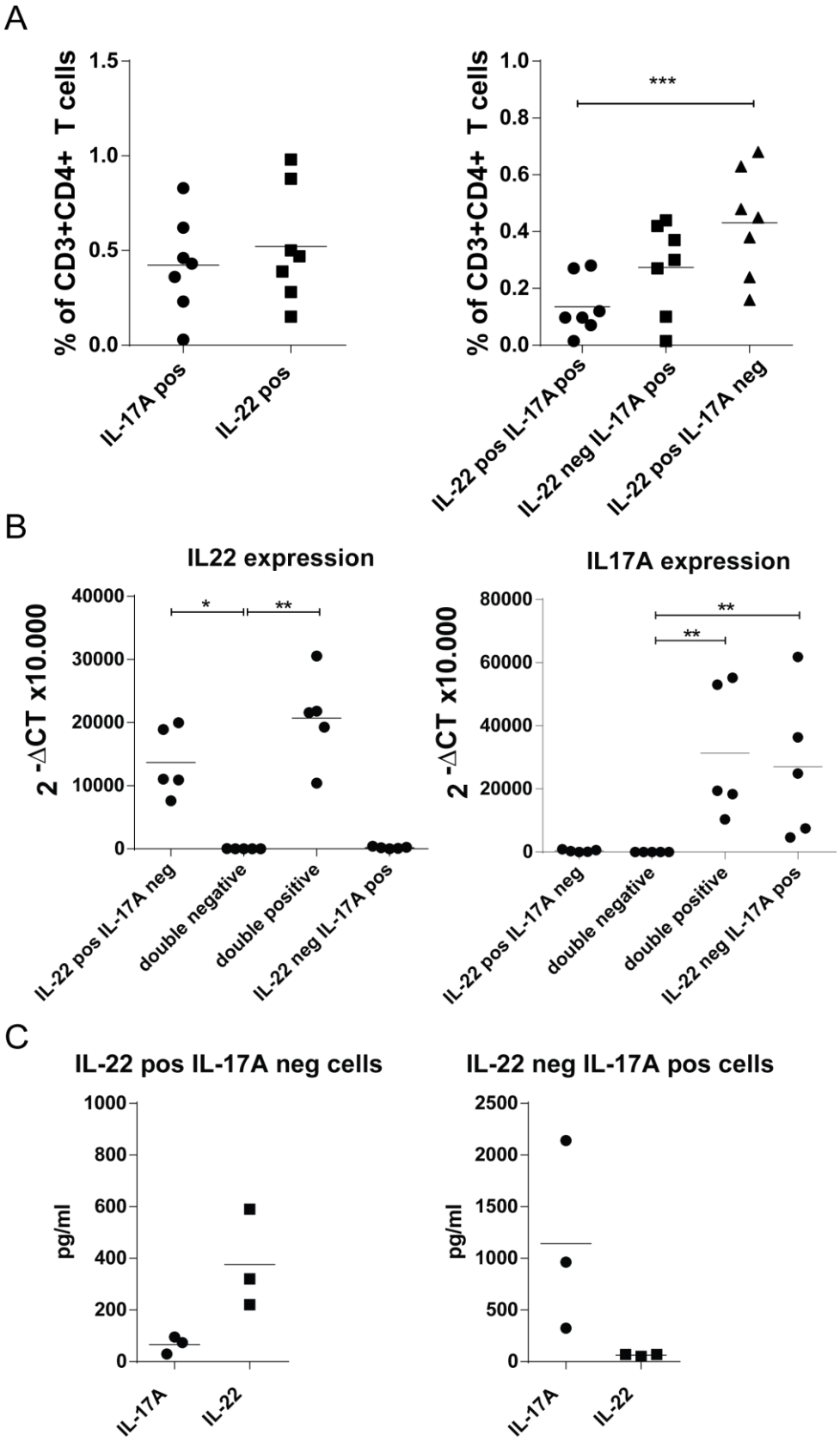

A
% of CD3+CD4+ T cells
1.5
1.0
0.5
0.0
IL-17A pos
IL-22 pos
% of CD3+CD4+ T cells
1.0
0.8
0.6
0.4
0.2
0.0
***
IL-22 pos IL-17A pos
IL-22 neg IL-17A pos
IL-22 pos IL-17A neg
B
IL22 expression
2 -ΔCT x10.000
40000
30000
20000
10000
0
*
**
IL-22 pos IL-17A neg
double negative
double positive
IL-22 neg IL-17A pos
IL17A expression
2 -ΔCT x10.000
80000
60000
40000
20000
0
**
**
IL-22 pos IL-17A neg
double negative
double positive
IL-22 neg IL-17A pos
C
IL-22 pos IL-17A neg cells
pg/ml
1000
800
600
400
200
0
IL-17A
IL-22
IL-22 neg IL-17A pos cells
pg/ml
2500
2000
1500
1000
500
0
IL-17A
IL-22

Figure 4

Supplementary figure legends

Supplementary figure 1. The principle of cytokine secretion assay.

Biotinylated cells were stained with streptavidin-labeled anti-cytokine catch antibodies. Next, cells were kept on the tube rotator in a 37^{o}C incubator and secreted cytokines were bound to the catch antibodies. In the last step, cytokines bound to the catch antibodies were detected by fluorescently labelled anti-cytokines antibodies.

Supplementary figure 2. Gating strategy for assessment of biotinylated and non-biotinylated cells proliferation.

After gating in lymphocytes, doublet cells were excluded and all viable cells were included into analysis. CD3+ T cells were separately gated from CD19+ B cells and their proliferation was analysed. A representative gating strategy shows cells stimulated with anti-CD2, CD3, CD28 mAb`s mixture for 6 days.

Supplementary figure 3. Validation of cytokine-secretion assay formation - graphical explanation.

Biotinylation of cell surface proteins was detected with fluorescently labelled streptavidin. In the next step, anti-cytokine catch antibodies were used to stain the cells. To detect the presence of catch antibodies on cell surface, goat anti-rat AF488 antibody and goat anti-mouse AF633 antibody were used to detect rat anti-human IL-22 Ab and mouse anti-human IL-17A Ab, respectively.

Supplementary figure 4. Detailed staining for representative donors in intracellular staining and secretion assays.

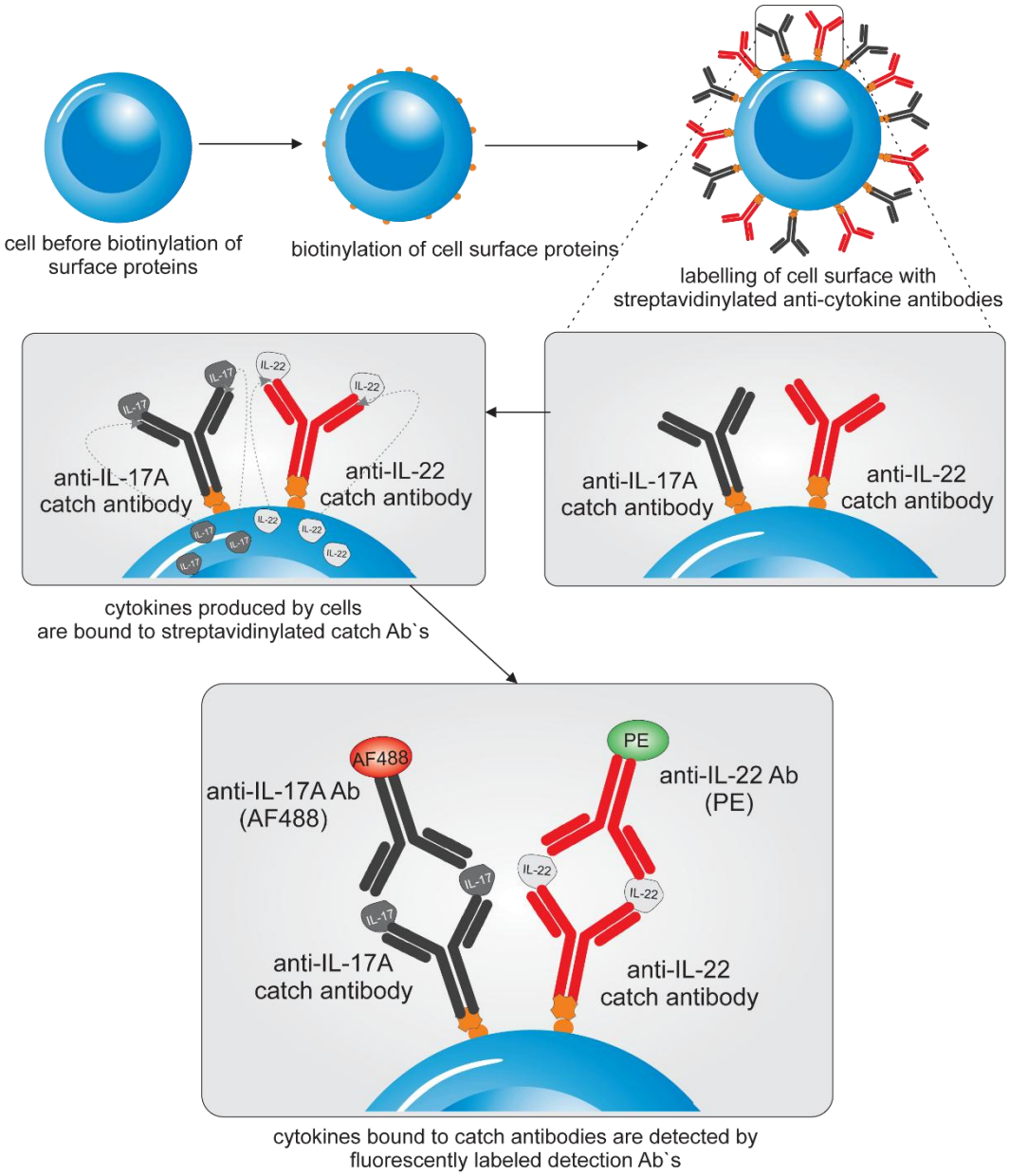
Detailed information about percentage of cells obtained from 2 donors (high and low cytokine producers) in secretion assay staining (A) and in intracellular staining (B). Gating strategy includes all cells based on forward scatter (FSC) and site scatter (SSC) and excludes dead cells using eFluor780 viability dye. Next, CD3+CD4+ T cells were investigated for expression of IL-17A, IL-22 or co-expression of both cytokines.

Supplementary figure 5. Protocol for staining IL-22- and IL-17A-producing CD3+CD4+ T cells with cytokine secretion assays.

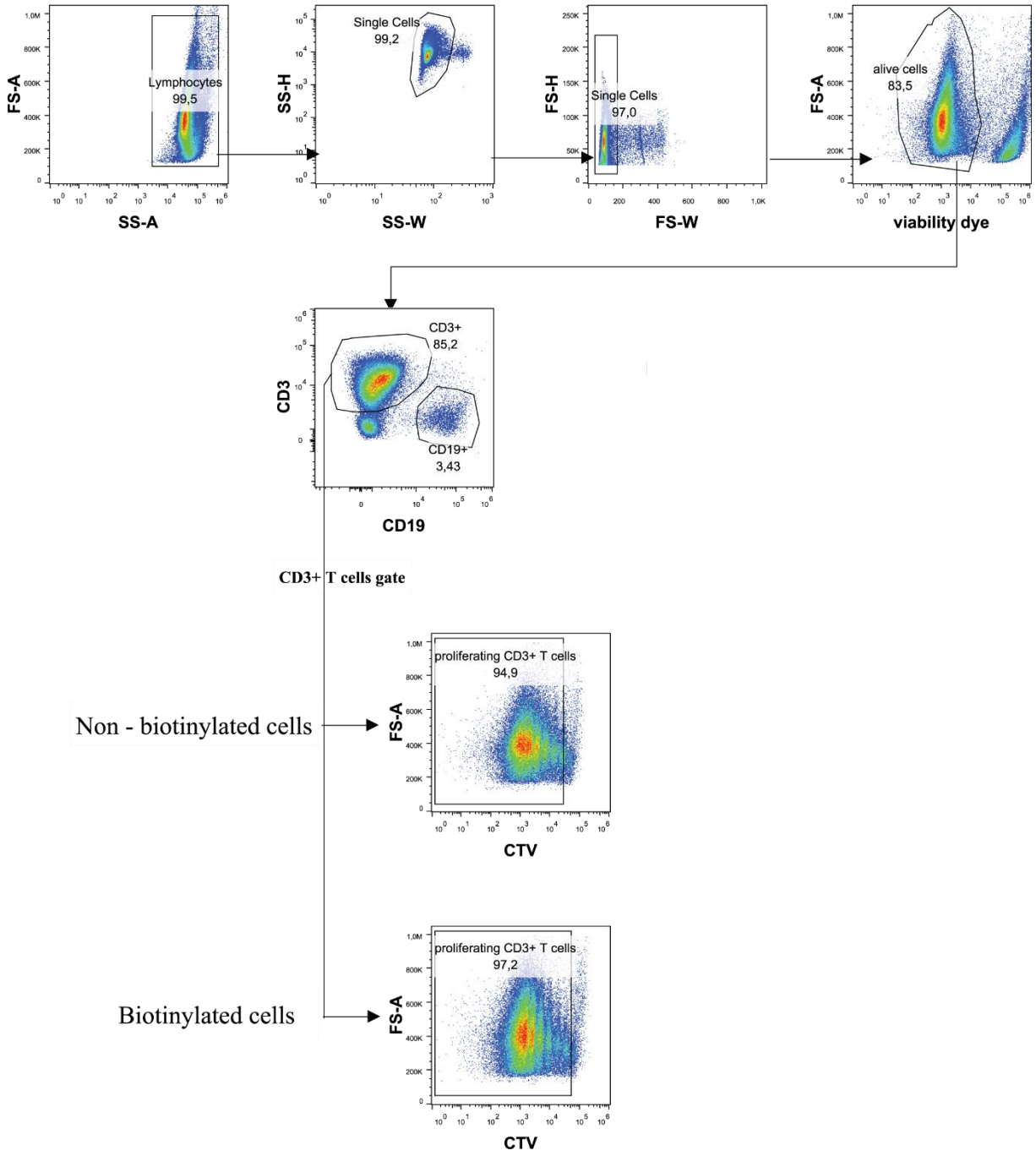
Detailed protocol of staining, which allows replication of data obtained in the presented paper.

Supplementary figure 6. Phenotypic differences between IL-22^{pos}IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells.

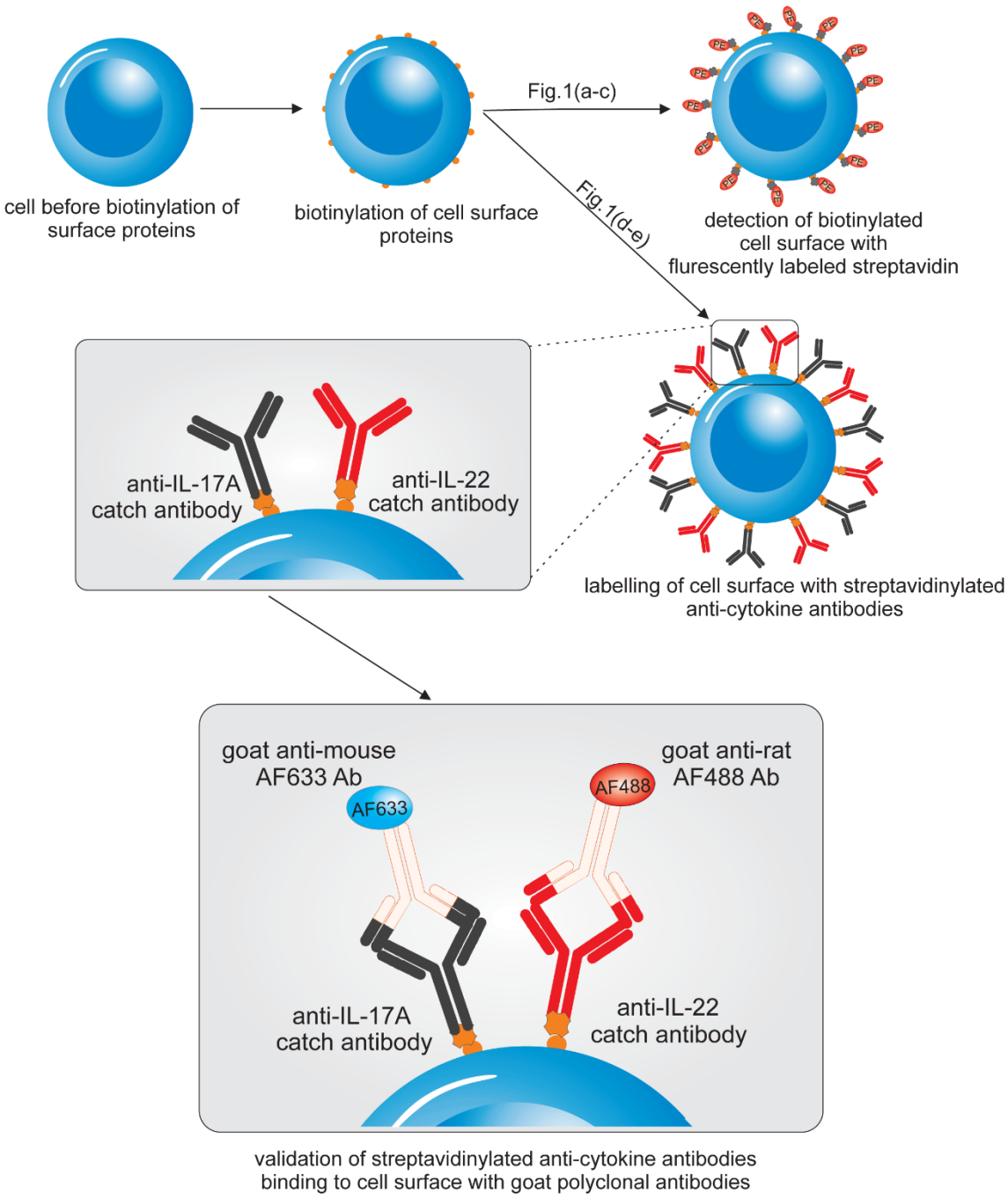
mRNA expression of chemokine receptors (CCR10, CCR6, CXCR3, CCR4), skin homing marker - CLA, transcription factors (T-bet, Gata-3, RORgt, AHR) and cytokines (IFN-γ, IL-4) was analyzed in IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells. Mann Whitney test was applied (* $p<0.05$, ** $p<0.005$).



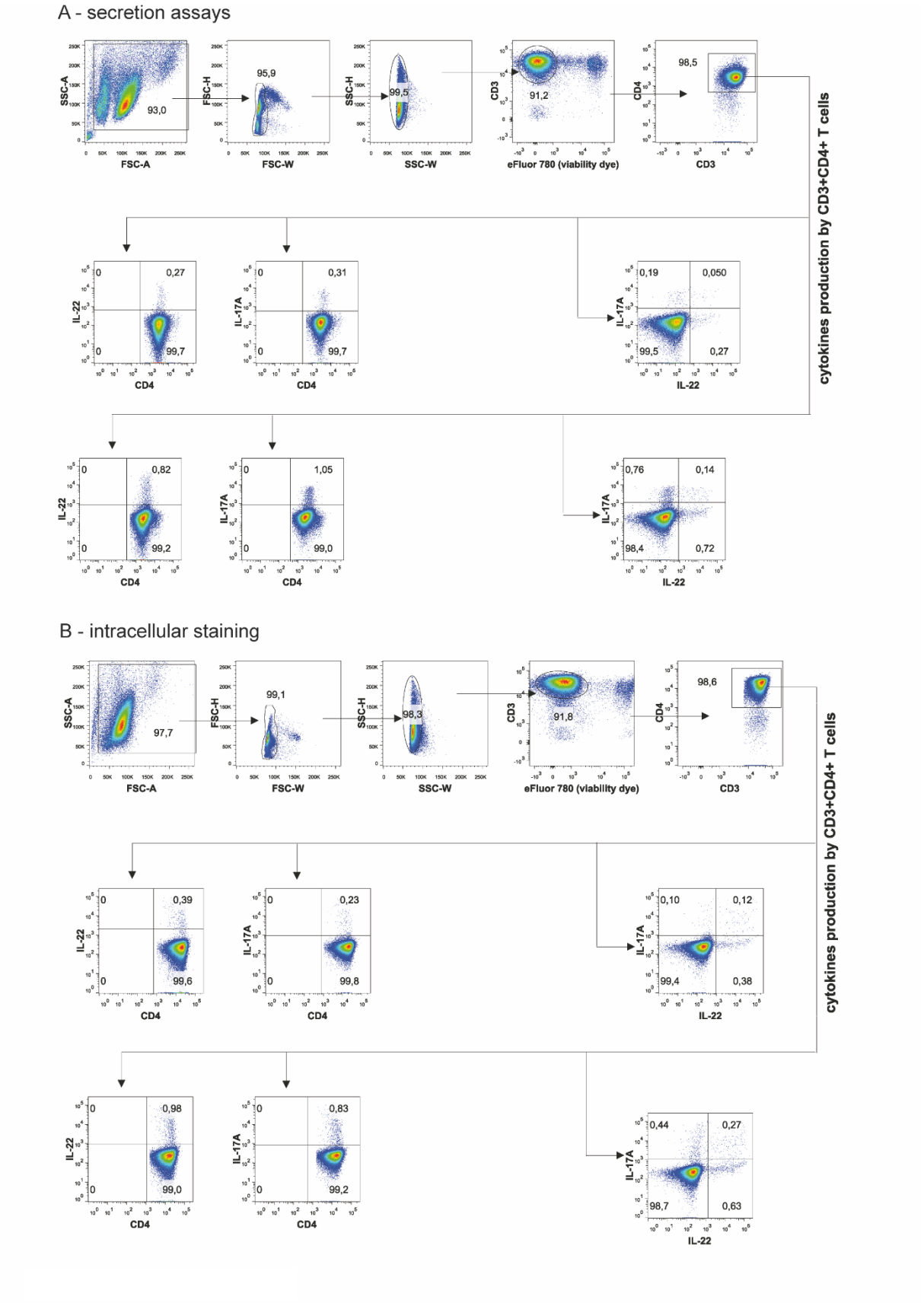
Supplementary Figure 1



Supplementary figure 2



Supplementary Figure 3



Supplementary Figure 4

Overview of protocol for staining IL-22 and IL-17A producing CD3+CD4+ T cells with cytokine secretion assays.

1) Labeling cell surface proteins with biotin

- Collect cells from cell culture [1]
- Wash cells 3 times with PBS pH 8.0 (300xg, 7 min)
- Divide cells for final concentration of 10 x 10^6 per tube
- Add 1ml of 1mg/ml biotin solution to each tube
- Mix and incubate for 30 min at room temperature
- Wash cells 3 times with PBS + glycine(100 nM) (300xg, 7 min)

2) Labeling of biotinylated cells with anti-cytokine catch antibodies

- Wash cells with staining buffer * (300xg, 7 min)
- Stain cells with 10 µg/ml of anti-IL-22 and anti-IL-17A catch antibodies, 5µl (0,25ug) of CD3 and 5µl (1ug) of CD4 antibodies premixed together in the final volume of 100 µl staining buffer
    - include controls, which do not contain anti-cytokine catch antibodies ("no-catch antibody control")
- Incubate cells for 20 min at 4-8°C
- Wash cells with staining buffer (300xg, 7 min)

3) Capture of cytokines secreted from cells by anti-cytokine catch antibodies

- Resuspend cells in pre-warmed at 37°C medium for final concentration of 1x10^6 cells/ml [2]
- Incubate cells on tube rotator in 37°C incubator for 4 h [3]
- Wash cells (300xg, 7 min)
- Wash cells with staining buffer (300xg, 7 min)

4) Detection of cytokine producing cells with anti-cytokine fluorescently labeled antibodies

- Stain cells with 10 µl of anti-IL-22PE (12 µg/ml) and 10 µl of anti-IL-17A AF488 (50 µg/ml) antibodies in total volume of 100 ul staining buffer
- Incubate cells for 20 min at 4-8°C
- Wash cells with staining buffer (300xg, 7 min)
- Just before sorting, stain cells with 5µl (0,25ng) of 7AAD viability dye for 5 min
- Resuspend cells in PBS and proceed for FACS sorting of the populations of interest.

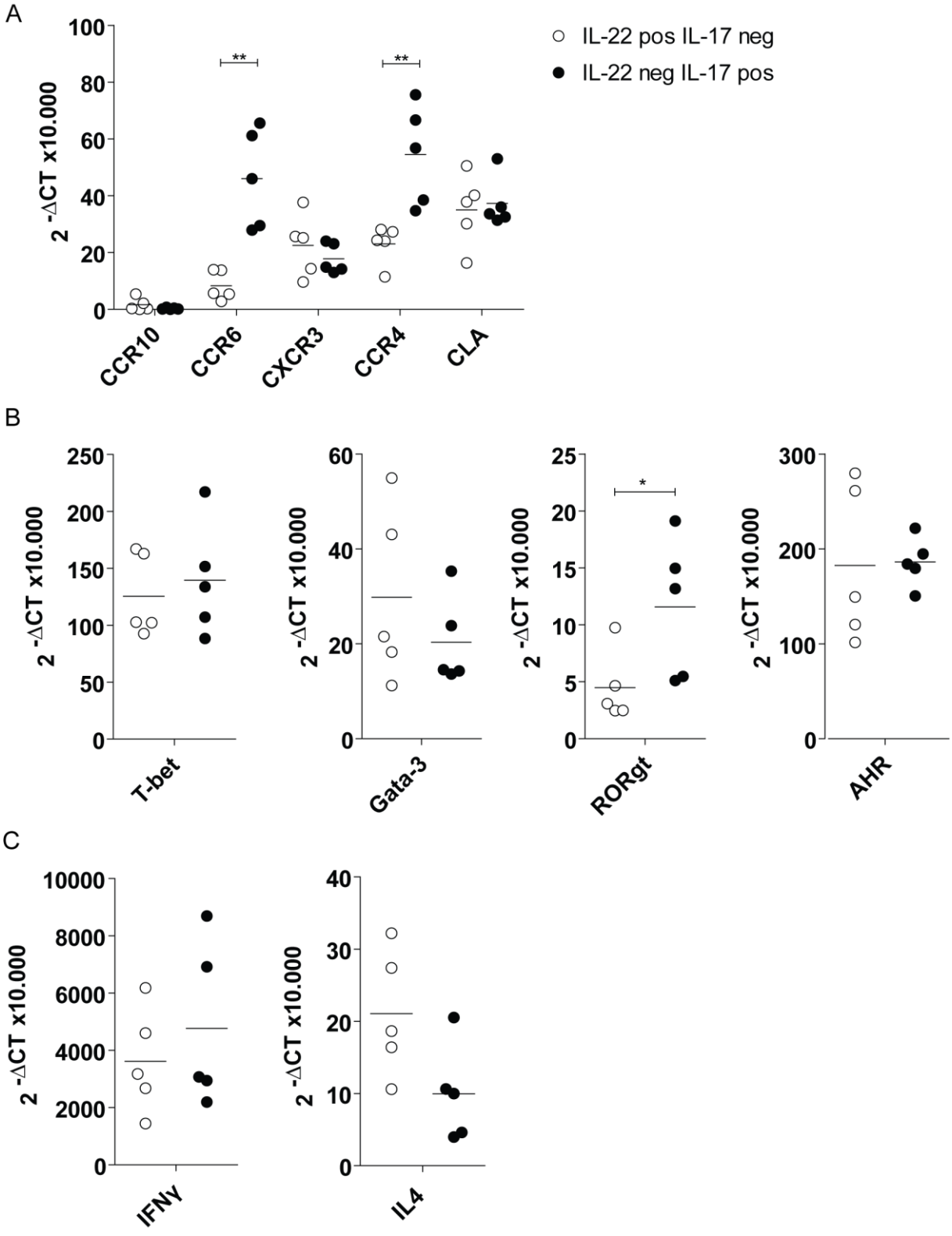
[1] To reduce amount of reagents used to isolate cytokine producing T helper cells, it is recommended to start whole staining procedure with pre-isolated CD3+CD4+ T cells.

[2] To minimalize probability of non-specific binding of cytokines to catch antibodies on the surface of cells which have not produced cytokines, cells are diluted to concentration 1x10^6 cells/ml medium.

[3] For the purpose of this presented study cells were stimulated with PMA/Ionomycin to resemble stimulation used in case of intracellular cytokine staining.

* staining buffer contains: PBS + 0.5% BSA + 2mM EDTA

Supplementary Figure 5



Supplementary Figure 6

Supplementary table 1.

Primers sequences used in analysis of phenotypic differences between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} sorted T cells.

Gene		Primer sequence
CCR10	forward	5` GAGGCCACAGAGCAGGTTTC 3`
	reverse	5` GGGAGACACTGGGTTGGAAG 3`
CCR6	forward	5` GAGAGGGCCCACGTGTATATG 3`
	reverse	5` ATTGATTCCCCGCTCATTGTG 3`
CXCR3	forward	5` GAAGTACGGCCCTGGAAGAC 3`
	reverse	5` CGGCGTCATTTAGCACTTGG 3`
CCR4	forward	5` AAAGCAAGCTGCTTCTGGTTG 3`
	reverse	5` AGGAAGAGCTCCCCAAATGC 3`
CLA	forward	5` GGCTGGGACCTTGTCACTAA 3`
	reverse	5` TCAGCAACAGGAGGAGTTGC 3`
T-bet	forward	5` GCAGGGACGGCGGATGTT 3`
	reverse	5` CAAATGAAACTTCCTGGCGCA 3`
Gata-3	forward	5` GCGCCGTCTTGATACTTTCAG 3`
	reverse	5` GGGTCACCTGGGTAGCGAAG 3`
Rorγc	forward	5` AGCTAGGTGCAGAGCTTCAG 3`
	reverse	5` ATTTGTGTTCTCATGACTGAGCC 3`
AHR	forward	5` TGGTTGTGATGCCAAAGGAAG 3`
	reverse	5` AAGCAGGCGTGCATTAGACT 3`
IL4	forward	5` ACA GCC TCA CAG AGC AGA AGA CT 3`
	reverse	5` GTG TTC TTG GAG GCA GCA AG 3`
IFNγ	forward	5` TCT CGG AAA CGA TGA AAT ATA CAA GTT AT 3`
	reverse	5` GTA ACA GCC AAG AGA ACC CAA AA 3`

6.4 Contributions to publications

For the publication entitled: “Increased microRNA-323-3p in IL-22/IL-17-producing T cells and asthma: a role in the regulation of the TGF-β pathway and IL-22 production”, I have contributed to experiments for figure 1 and figure 2.

For the publication entitled: “Next generation sequencing characterization of human IL-22-producing T cells”, I have contributed to all experiments except the performance of sequencing and analysis of next generation RNA sequencing data.

For the publication entitled: “A novel, dual cytokine-secretion assay for the purification of human Th22 cells that do not co-produce IL-17A”, I have contributed to all experiments.

7 General discussion

T cells are essential cells of the adaptive immune system. Due to their highly specific and diverse repertoire of antigen recognizing T cell receptors on the surface, T cells can eliminate viral, fungal and bacterial infections. Additionally, by releasing cytokines, T cells activate other immune cells, assist B cells to produce antibodies and regulate the immune system and tissue cells. After antigen recognition during the immune synapse formation and delivery of three signals by antigen-presenting cells, CD4+T cells differentiate into effector cells that are characterized by subset inducing polarizing cytokines, lineage-specific transcription factors and a range of cytokines produced to convey effector function. The main subsets of effector T helper cells are: Th1, Th2, Th9, Th17 and Th22 cells. Among all of these T helper cells subsets, IL-22-producing T cells have been characterized to a lesser extent compared to other subsets. In this PhD thesis, we have studied human IL-22-producing T cells in depth and increased the knowledge about human IL-22-producing T cells.

MicroRNAs are post-transcriptional gene expression regulators, which play a role in the development and effector functions of T cells. For example miR-146 and miR-155 enhances Th1-mediated immunity [269, 270]. On the contrary, miR-29a and miR-29b are the most potent direct inhibitors of Th1 differentiation and IFN-γ production [265]. Additionally, polarization of naive T cells towards Th17 cells is enhanced mainly by miR155, miR-301 and miR-326 [278-280, 283]. Nevertheless, miRNA profiling and miRNA functional analysis in IL-22-producing T cells have not been addressed so far.

For the first time, we have analyzed the expression of microRNAs in human IL-22-producing T cells and demonstrated that IL-22-producing T cells have a distinct miRNA expression pattern. A total of 18 miRNAs were found to be differentially expressed with more than 2-fold difference in $CD3^{+}IL\text{-}22^{+}$ compared to $CD3^{+}IL\text{-}22^{-}$ T cells. miR-323-3p, miR-886-5p, and miR-886-3p

were significantly up-regulated and miR-874, miR-26a, miR-181a, miR-93, and miR-146a down-regulated in $CD3^+IL\text{-}22^+$ cells. Additional analysis confirmed, that among $CD3^+IL\text{-}17^-IL\text{-}22^+$, $CD3^+IL\text{-}17^+IL\text{-}22^-$, $CD3^+IL\text{-}17^+IL\text{-}22^+$ and $CD3^+IL\text{-}17^-IL\text{-}22^-$, the expression of miR-323-3p and miR-886-5p in $CD3^+IL\text{-}17^+IL\text{-}22^+$ cells was increased in comparison to the cells, which did not produce these cytokines.

Pathway analysis of miRNAs differentially expressed in IL-22-positive T cells suggested that these miRNAs might impact the development, proliferation and immune responses of T cells. miR-886-5p and miR886-3p were excluded from the above mentioned pathway analysis as the corresponding PCR probes were recently shown to recognize a 139 nt long noncoding RNA nc886 that functions as a repressor of interferon-induced, double-stranded RNA-activated protein kinase [302]. Integrated pathway and target analysis suggested that miR-323-3p could target and modulate the expression of the genes regulating cellular responses to TGF-β, which is known to play a crucial role in Th17 cells differentiation and regulation of IL-22 production by Th17 cells [145].

SMAD3, SMAD5 and CDKN1B were determined as direct miR-323-3p targets with SMAD3 and CDKN1B being inhibited by the transfection of miR-323-3p mimics into T cells. As it is known that SMAD3 and SMAD5 are able to translocate to the nucleus, where together with transcriptional activators and repressors they modulate gene expression [305] and CDKN1B controls the cell cycle progression at G1 [306], we propose that miR-323-3p might impact the progression of the cell cycle and the expression of cytokines. Interestingly, strong down-regulation of IL-22 mRNA level as well as its protein level was observed, when T cells were transfected with miR-323-3p.

Although miR-323-3p targets several genes from the TGF-β pathway, which is an important cytokine needed for Th17 cell differentiation which suppress the production of IL-22 [145]. At the same time miR-323-3p

cooperates with TGF-β in the suppression of IL-22 production. One of the possible explanations of this finding is that miR-323-3p may regulate TGF-β-related genes other than the ones investigated in this study. Nevertheless, this data, for the first time shows that miRNA-323-3p acts as a negative regulator of IL-22 production by T cells.

IL-22 may be produced by Th22 and Th17 cells, therefore IL-22-producing T cells consist of a mixture of IL-22-single and IL-22/IL-17 co-producing T cells. In our study, we show that the expression of miR-323-3p is higher in IL-22/IL-17-double positive T cells and IL-17-single positive T cells as compared to IL-22-single positive T cells. This observation may suggest that higher levels of miR-323-3p inhibit IL-22 production and thus shift IL-22/IL-17 co-producing T cells towards the Th17 phenotype that lack IL-22 production. As IL-22 is known as bi-functional cytokine that acts differently on epithelial cells depending on the cytokine milieu [345], miR-323-3p may play a role as a potential regulator of IL-22 production that may influence the final effect of Th17/Th22 cells on tissues.

Recently published studies suggest that both Th17 and Th22 cells play an important role in allergic diseases [290, 291, 346]. For example, Th17 cells have been demonstrated to play a central role in neutrophilic airway inflammation [290, 291]. Additionally, in mouse models, IL-22 has been shown to suppress recruitment of eosinophils and goblet cell hyperplasia in antigen-induced airway inflammation [194]. Moreover, in the serum of patients with asthma, levels of both IL-22 and IL-17 have been shown to be increased [190, 313]. In agreement with this data, increased expression of miR-323-3p in PBMC`s from patients with asthma was observed when compared to control, healthy individuals, suggesting that altered expression of miR-323-3p may potentially impact immune responses in allergic diseases. However, further studies about the role of miR-323-3p in IL-22/IL-17-producing T cells in asthma need to be conducted.

In conclusion of first part, we show the increased expression of miR-323-3p in IL-22/IL-17-producing T cells and in PBMCs from patients with asthma compared to healthy controls. In addition, we demonstrate that miR-323-3p targets multiple genes from the TGF-β signaling pathway and inhibits IL-22 production by T cells. These results suggest that miR-323-3p might impact T cell responses in asthma.

Considering the fact that IL-22 plays a unique role in the regulation of epithelial cells, next we characterized human IL-22-producing T cells from palatine tonsils, where T cells are in the close proximity to epithelial cells. Viable human IL-22-producing T cells were isolated from palatine tonsil mononuclear cells (TMCs) with the use of an in-house generated IL-22-secretion assay followed by next generation sequencing of IL-22-positive and IL-22-negative T cells.

In the first steps, to better characterize IL-22-producing T cells from tonsils, we used intracellular cytokine staining for Th1/Th2/Th17/Th22/Treg cytokines (IL-17, IL-10, IL-4, IL-13, IFN-γ, IL-22). Interestingly, co-expression analysis of IL-17, IL-10, IL-4, IL-13 and IFN-γ by IL-22-producing $CD4^+$ T cells revealed that most of IL-22-producing cells either co-express IL-4 only or co-express IL-17 only or produce IL-22 alone. Furthermore, when similar analysis was performed for IL-17-producing, IL-4-producing, IFN-γ-producing and IL-10-producing T cells, percentage of IL-17-single positive, IL-10-single positive, IL-4-single positive or IFN-γ-single positive cells was statistically higher when compared to IL-22-single producing T cells. Since the effect of IL-22 on epithelial cells may depend on other cytokines, in this situation IL-4 and IL-17, this results support the common understanding that IL-22 is a bifunctional cytokine, which may play both a protective and pro-inflammatory role on epithelial cells depending on cytokine environment.

Later, we established that TLR3 ligand (Poly (I:C)), IL-7 and IL-23 stimulation of TMCs induced high IL-22 production on mRNA as well as on protein level. After analyzing other TLR ligands and cytokines, this is the first report of this set of stimuli being able to induce IL-22 production. It is possible that TLR3 ligand, Poly (I:C), via activation of STAT3 signaling induces IL-22 [347]. At the same time, IL-7 serves as a survival factor [348] and IL-23 induces direct IL-22 production [349]. However, the mechanisms of IL-22 induction by the stimuli of TLR3 ligand, IL-7 and IL-23 remain to be explained thoroughly.

Because of the fact that anti-IL-22 secretion assay is not commercially available, we generated it in our laboratory. Anti-CD45 antibody was conjugated with anti-IL-22 antibody to form an anti-IL-22 catch reagent that was used in the first step of IL-22 secretion assay. During the validation of catch reagent formation, we show that all white blood cells have been stained with anti-IL-22 catch reagent. Next, before performing next generation RNA sequencing analysis, we proved that cells sorted according to IL-22 production express high levels of IL-22 mRNA and during long term culture keep the profile of IL-22-producing cells. So far, most of the attempts to characterize IL-22-producing T cells were based on intracellular cytokine staining, generation of Th22 cell clones or sorting the IL-22-positive cells according to expression of chemokine receptors [147, 287, 288]. The method, which we present here in this thesis is novel and for the first time allows to sort viable, human IL-22-producing T cells.

Next generation RNA sequencing analysis of IL-22-producing and IL-22-non-producing T cells revealed 57820 genes differentially expressed in IL-22-positive cells compared to IL-22-negative cells. List of 601 genes that fulfilled the criteria of: genes expressed in at least 3 out of 4 donors in either IL-22-positive or IL-22-negative T cells with the intensity values above 0, with a log2 value ratio ≥ 1 or ≤ -1 and differently expressed with the p Value below 0.01 has been included in the analysis with the MetaCore-Data-mining and pathway analysis software. 13 genes recognized as coding receptor ligands (cytokines),

13 genes as coding transcription factors and 6 genes as chemokine receptors (G protein-coupled receptors) have been identified within the list as differentially expressed between IL-22-positive and IL-22-negative T cells.

Among the cytokines listed, IL-22-producing T cells express higher levels of IL-22, IL-17A, IL-17F, IL-1A, IL-9, IL-21, IL-26 and CCL1, CCL2, CCL20. As IL-17A and IL-17F production by IL-22-positive T cells have been described previously [141], other molecules are reported for the first time to be co-expressed together with IL-22. This data suggests that IL-22-producing T cells via IL-22 production may influence cells by the production of other cytokines and chemokines and exhibit a broader effect of the immune system. As IL-22 is known to be bi-functional, depending on the cytokine milieu [324], it remains unclear what would be the effect of IL-22 on the immune cells in the context of the above mentioned co-produced cytokines. At the same time, IL-22-producing T cells differentially express CCR5, CXCR4 and CXCR6 that may be used as surface markers for IL-22-producing cells identification. Interestingly, CCR5 is described as inflammatory chemokine receptor [323]. CCR5 is expressed by Th1 cells. However more is known about the expression of CCR5 on Treg cells, where it is critical for the migration to tissues infected with fungal pathogens [325] and *Leishmania major* [326]. Additionally, during graft-versus-host disease, CCR5 mediates accumulation of Tregs in graft-versus-host disease targeted organs [327]. In the islet allograft transplant model, Treg cell migration from blood to inflamed transplant tissue requires CCR5 expression [328]. Similar role in related to migration of cells to tissues was found for CXCR6, which promote NKT cells residence in peripheral tissues like liver and lung [329]. In contrast, another chemokine receptor expressed by IL-22-positive T cells - CXCR4, mediate CCR7-independent entry to lymph nodes [330]. It is interesting that chemokine receptors expressed differentially on IL-22-positive cells may guide the migration of IL-22-postive cells to inflamed tissue, where by IL-22 production they may influence tissue epithelial cells.

Finally, Th22 cells-related transcription factor was not described previously and we show for the first time among the potential transcription factors upregulated in IL-22-producing T cells - PPARG and among transcription factor downregulated in IL-22-producing T cells - FOS, bind directly to IL22 promotor and may influence the IL-22-production directly [SABiosciences` proprietary database]. Role of other transcription factors upregulated (MAF, EPAS1, MSC, CITED) and downregulated (EGR1, EGR4, ZXDA) in IL-22-positive T cells has to be investigated further.

In summary, present study for the first time shows the next generation sequencing analysis of human IL-22-producing T cells and proposes the genes that characterize human IL-22-producing T cells. Further investigation of biological function of those molecules in IL-22 secreting T cells is required. Additionally, there is certainly a need to distinguish between IL-22-producing Th17 cells and IL-22-producing Th22 cells and better characterize pure human Th22 cells.

The main cytokine of Th22 cells, IL-22, can be also produced by Th17 cells and therefore there is still an ongoing debate and no clear distinction between Th17 and Th22 cells in humans. Most of the confusion in the assigning IL-22 as Th17 rather than Th22 cytokine comes from a discrepancy between mouse and human data. Among the mouse T helper subsets, Th17 cells are the major source of IL-22 and IL-22 is named as a Th17 cytokine [141-143]. In contrast, IL-22 production by human cells does not correlate with either RORγt or IL-17A expression [139] and only 10-18% of IL-22-producing T cells in blood co-express IL-17A [287]. To improve the current methodology that enables better characterization of human IL-22-producing T cells, we developed a novel, dual cytokine secretion assay to purify human Th22 cells that do not co-produce IL-17A. So far, the commercially available secretion assays consists of an anti-cytokine catch reagent (anti-CD45 Ab linked to an anti-cytokine Ab) and

fluorescently labelled detection Ab that binds to a different epitope of the cytokine of interest. One of the main restrictions of those secretion assays is limited number of fluorescently labelled detection antibodies that exist and the fact that the IL-22-secretion assay has not been developed so far. On the contrary, our newly developed cytokine secretion assay consists of anti-IL-22 and anti-IL-17A catch antibodies, which via a biotin-streptavidin interaction are bound to the biotinylated surface of the target cell and anti-IL-22 and IL-17A detection antibodies labelled with a fluorescent dye, which detects cytokines bound to these catch antibodies.

The secretion assay presented in this thesis is an improved version of secretion assay used for the next generation sequencing analysis of human tonsil T cells. As we faced technical problems to combine two secretion assay that would detect IL-22 and IL-17 simultaneously, we decided to change the main principle of the methodology and instead of using catch reagent consisting of anti-CD45 conjugated to anti-cytokine catch reagent, we used streptavidin-labelled anti-cytokine antibody that binds to the biotinylated cell surface.

Importantly, the biotinylation of cell surface proteins that takes place in the currently used dual-secretion assay did not affect the viability and ability of cells to proliferate and did not activate the cells. These results suggest that biotinylated and sorted cells can be further cultured *in vitro.* In the subsequent step, anti-cytokine antibodies labelled with streptavidin were used to detect and bind cytokines produced by cells. This step opens new possibilities, since any purified anti-cytokine antibody can be labelled with streptavidin and used as anti-cytokine catch antibody as long as fluorescently labelled detection antibodies used in next step recognize different epitopes of the same cytokine. Obviously, with multiple-anti-cytokine secretion assay`s combined, this methodology enables one to analyze more than two cytokines at the same time. Especially, this method may be useful for hard do detect cytokines, for which cytokine secretion assays have not yet been developed commercially.

The validation of dual-secretion assay required comparison with intracellular staining of cytokines in terms of percentage of cells detected with both methods. In the present study, the percentages of cells detected with intracellular staining significantly correlated with the percentages of cells detected with the dual-secretion assay, suggesting that secretion-assay developed by us is as sensitive as intracellular cytokine staining for the detection of cytokine-producing cells.

Finally, cells sorted with dual-secretion assay, namely Th17 cells that produce IL-17A and do not co-express IL-22 and Th22 cells that produce IL-22 and do not co-express IL-17A, were characterized in terms of transcription factors and chemokines receptors expression. Higher levels of mRNA expression of CCR6 and CCR4 were detected in IL-22^{neg} IL-17^{pos} T cells as compared to IL-22^{pos} IL-17^{neg} cells, suggesting that the previously reported pattern of chemokines receptors expression - CCR6^{+}CCR4^{+}CXCR3^{-} [287, 288] better describes Th17 rather than Th22 cells. Similarly, IL-22^{neg} IL-17^{pos} expressed higher levels of RORC2 transcription factor compared to IL-22^{pos} IL-17^{neg} T cells. Although, aryl hydrocarbon receptor (AhR) was expressed in relatively high levels in both cell subsets and the expression was not different between IL-22^{pos} IL-17^{neg} and IL-22^{neg} IL-17^{pos} cells in the present study. Therefore this might imply what has been reported previously that AhR is not required for IL-22 expression in T cells [145].

In summary, this novel method expands the possibilities to further characterize human Th22 cells that may be co-cultured with other cells or used in humanized mouse models. Additionally, the method may be used to identify and sort other immune cell subsets that are characterized by the production of cytokines.

Conclusion and outlook

We conclude here that the data demonstrated in these studies is novel in terms of three aspects. First of all, for the first time miRNA expression profile analysis in human IL-22-producing T cells has been performed and a new miRNA-323-3p has been reported as a potential negative regulator of IL-22 production from T cells. Secondly, next generation sequencing of human IL-22-producing T cells sorted with our in-house generated novel IL-22 secretion assay was performed and a full profile of IL-22-producing cells including potential transcription factors was demonstrated. Finally, a novel, double cytokine secretion assay that allows sorting of viable Th22 cells that do not co-produce IL-17A was generated.

The novel methods developed here will enable better understanding and characterization of human Th22 cells may provide novel therapeutic options in the treatment of diseases like psoriasis, atopic dermatitis, rheumatoid arthritis, where pro-inflammatory role of IL-22 was reported.

8 Curriculum vitae

Personal data

Name:	Marcin
First name:	Wawrzyniak
Address:	Promenade 127
	7260 Davos Dorf
	Switzerland
Date of birth:	21.06.1983
Place of birth:	Lodz, Poland
Nationality:	Polish
Marital status:	Married
Phone :	+41 779 48 98 11
Email:	marcin.wawrzyniak@siaf.uzh.ch

Education and Experience

01.2010 – 11.2015	PhD student at Swiss Institute of Allergy and Asthma Research, Davos, Switzerland
10.2009 – 01.2015	Stipendium in Swiss Institute of Allergy and Asthma Research, Davos, Switzerland
09.2008 – 10.2009	Scientific Assistant in Department of Laboratory Medical Immunology, Medical University of Lodz, Lodz, Poland
09.2007 – 08.2008	Microbiologist in Norbrook Laboratories Ltd, Research & Development Department, Newry, Northern Ireland
2002 - 2007	Master of Science Degree in Biology,
	Specialization: Microbiology and Immunology,
	University of Lodz, Faculty of Biology and Environmental Protection, Institute of Cellular Immunology, Lodz, Poland
	Practice during MSc Study:
	Holiday practice in microbiological laboratory in "Maria Konopnicka, University Clinical Hospital No.4", Lodz, Poland
	Holiday practice in "Sanitary Epidemiological Service", Lodz, Poland
	Holiday practice in microbiology laboratory in "JOGO" creamery, Lodz, Poland

Awards and Honors

Best Presentation Award – SIAF Science Day - 2013, Davos, Switzerland

Abstract Prize Winner – EAACI-WAO Congress 2013, Milan, Italy

Examinations and Courses during PhD

11.03 – 12.03.2010	Cell Sorting Course, SIAF, Davos
06.06 – 10.06.2010	5th Microbiology and Immunology Introductory Course, Zurich
17.11 – 18.11.2011	Laboratory Methods: Molecular and Cell Biology
24.01 – 02.02.2011	LTK Module 1: Introductory Course in Laboratory Animal Science, Zurich
15.01 – 20.01. 2012	Microscopy Winter School 2012 – Practical Course in Advanced 3D microscopy, Zurich
04.04 + 18.04.2012	Voice Training and Presentation Skills Course, Zurich
2010 – 2011 & 2014-2015	Immunology Lectures, SIAF, Davos
19.05 – 23.05.2014	RNA-sequencing and Transcriptomics Analysis Workshop, SIAF, Davos
17.02 - 19.02.2015	Bitplane Imaris - European User Group Meeting, Vienna, Austria

Presentations at SIAF

Progress Reports:	**Journal Clubs:**	**SIAF Science Day:**
2010.07.01	2010.05.11	2010.12.15
2010.11.19	2010.09.21	2011.12.14
2011.05.03	2011.02.22	2012.12.20
2011.11.22	2011.11.08	2013.12.19
2012.06.26	2012.05.06	2014.12.18
2012.11.28	2012.11.09	
2013.05.23	2013.06.04	
2013.11.28	2013.10.11	
2014.06.30	2014.07.15	
2014.11.11	2014.10.07	
2015.05.04	2015.07.07	

Presentations at Scientific Congresses

12.02 – 15.02.2012	10th EAACI GALEN Immunology Winter School – Basic Immunology research in allergy – Oral Presentation – Are, Sweeden
15.03 - 19.03.2012	World Immune Regulation Meeting VI – Poster Session – Davos, Switzerland
27.01 - 30.01.2013	11th EAACI GALEN Immunology Winter School – Basic Immunology Research in Allergy and Clinical immunology - Oral Presentation – Pichl, Austria
13.03 - 16.03.2013	World Immune Regulation Meeting VII – Poster Session – Davos, Switzerland
03.04 - 05.04.2013	XXV Meeting of the Swiss Immunology PhD students – Oral Presentation – Schloss Wolsberg, Switzerland
22.06 - 16.06.2013	EAACI Congress – Poster Session – Milano, Italy
19.04 - 22.04.2014	World Immune Regulation Meeting VIII – Poster Session – Davos, Switzerland
10.09 - 11.09.2014	Academia Reatica – Poster Session – Davos, Switzerland
18.03 - 21.03.2015	World Immune Regulation Meeting IX – Poster Session – Davos, Switzerland

Publications

van de Veen W., Stanic B., Yaman G., **Wawrzyniak M.**, Söllner S., Akdis DG., Rückert B., Akdis CA., Akdis M.,

IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses,

J Allergy Clin Immunol. 2013 Apr; 131(4):1204-12

Soyka MB., Holzmann D., Basinski TM., **Wawrzyniak M.**, Bannert C., Burgler S., Akkoc T., Treis A., Rückert B., Akdis M., Akdis CA. Eiwegger T.,

The Induction of IL-33 in the Sinus Epithelium and Its Influence of T-Helper Cell Responses,

PLOS One, 2015, May 1:10 (5)

Wawrzyniak M., Ochsner U., Wirz O., Wawrzyniak P., van de Veen W., Akdis C.A., Akdis M.

A novel, dual cytokine-secretion assay for the purification of human Th22 cells that do not co-produce IL-17A.

Allergy, doi: 10.1111/all.12768

Kärner J., **Wawrzyniak M.**, Tankov S., Runnel T., Aints A., Kisand K., Altraja A., Kingo K., Akdis C. A., Akdis M. & Rebane A.

Increased microRNA-323-3p in IL-22/IL-17-producing T cells and asthma: a role in the regulation of the TGF-β pathway and IL-22 production

Manuscript under revision in Allergy

Wawrzyniak P., **Wawrzyniak M**., Wanke K., Sokolowska M., Bendelja K., Ruckert B., Globinska A., Jakiela B., Kast J., Idzko M., Akdis M., Sanka M., Akdis C.A.

Regulation of bronchial epithelial barrier integrity by type 2 cytokines and histone deacetylases in asthma.

Manuscript under revision in J Allergy Clin Immunol.

Wawrzyniak M., Wawrzyniak P., Breedveld A., Rebane A., Morita H., Rückert B., Castro Giner F., Rhyner C., Akdis C., Akdis

Transcriptomic characterization of human IL-22-producing T cells by next generation RNA sequencing

Manuscript in preparation

Wawrzyniak P., Terufumi K., Altunbulakli C., Kazunari S., **Wawrzyniak M.**, Akdis M., Akdis C.A.

Regulation of Epithelial Barrier in Asthma

Proceeding of the 30th Symposium of the Collegium Internationale Allergologicum – awaiting publication

Mübeccel Akdis MD, PhD, Alar Aab MSc, Can A1tunbulaklii MSc, Kursat Azkur MD, Rita A. Costa MSc, Reto Crameri PhD, Su Duan MD, Thomas Eiwegger MD, Ruth Ferstl PhD, Remo Frei PhD, Mattia Garbani MSc, Anna G1obinska MSc, Carly Huitema PHD, Terufumi Kubo MD, Zsolt Komlosi MD, Patricja Konieczna PhD, Nora Kovacs MD, Umut C. Kucuksezer PhD, Norbert Meyer MD, Hideaki Morita MD, Judith Olzhausen PhD, Liam O'Mahony PhD, Marija Pezer PhD, Moira Prati MSc, Ana Rebane PhD, Claudio Rhyner PhD, Arturo Rinaldi MSc, Barbara Stanic PhD Kazunari Sugita MD, Angela Treis PhD, Willem van de Veen PhD, Kerstin Wanke MSc, **Marcin Wawrzyniak MSc**, Paulina Wawrzyniak MSc, Oliver Wirz MSc, Josefina Sierra Zakzuk MD, Cezmi A. Akdis MD.

Interleukins, from 1 to 38, interferons, transforming growth factor-β and tumor necrosis factor-α: receptors, functions, and roles in diseases

Manuscript in preparation to J Allergy Clin Immunol

9 References

1. Owen, J., J. Punt, and S. Stranford, *Kuby Immunology*. 7th ed. 2012: MacMillan Education.
2. Murphy, K., *Janeway`s Immunobiology*. 8th ed. 2012: Garland Science, Taylor & Francis Group, LLC.
3. Abbas, A.K., L. A.H., and S. Pillai, *Cellular and Molecular Immunology*. 7th ed. 2012: Elsevier.
4. Shen, L., *Tight junctions on the move: molecular mechanisms for epithelial barrier regulation.* Ann N Y Acad Sci, 2012. **1258**: p. 9-18.
5. Kast, J.I., et al., *The broad spectrum of interepithelial junctions in skin and lung.* J Allergy Clin Immunol, 2012. **130**(2): p. 544-7 e4.
6. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* Int Rev Immunol, 2011. **30**(1): p. 16-34.
7. Hajishengallis, G. and J.D. Lambris, *Microbial manipulation of receptor crosstalk in innate immunity.* Nat Rev Immunol, 2011. **11**(3): p. 187-200.
8. Hashimoto, C., K.L. Hudson, and K.V. Anderson, *The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein.* Cell, 1988. **52**(2): p. 269-79.
9. Lemaitre, B., J.M. Reichhart, and J.A. Hoffmann, *Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14614-9.
10. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
11. Kumar, H., T. Kawai, and S. Akira, *Toll-like receptors and innate immunity.* Biochem Biophys Res Commun, 2009. **388**(4): p. 621-5.
12. O'Neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors - redefining innate immunity.* Nat Rev Immunol, 2013. **13**(6): p. 453-60.
13. Akashi-Takamura, S. and K. Miyake, *TLR accessory molecules.* Curr Opin Immunol, 2008. **20**(4): p. 420-5.
14. Kang, J.Y., et al., *Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer.* Immunity, 2009. **31**(6): p. 873-84.
15. Jin, M.S., et al., *Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide.* Cell, 2007. **130**(6): p. 1071-82.
16. Uematsu, S., et al., *Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5.* Nat Immunol, 2008. **9**(7): p. 769-76.
17. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.
18. Kawai, T. and S. Akira, *Toll-like receptor and RIG-I-like receptor signaling.* Ann N Y Acad Sci, 2008. **1143**: p. 1-20.
19. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses.* Nat Rev Immunol, 2014. **14**(1): p. 36-49.
20. Zhang, S.Y., et al., *TLR3 deficiency in patients with herpes simplex encephalitis.* Science, 2007. **317**(5844): p. 1522-7.
21. Tabeta, K., et al., *Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3516-21.
22. Kawai, T. and S. Akira, *Innate immune recognition of viral infection.* Nat Immunol, 2006. **7**(2): p. 131-7.
23. van de Veen, W., et al., *IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses.* J Allergy Clin Immunol, 2013. **131**(4): p. 1204-12.
24. Mansson, A., M. Adner, and L.O. Cardell, *Toll-like receptors in cellular subsets of human tonsil T cells: altered expression during recurrent tonsillitis.* Respir Res, 2006. **7**: p. 36.

25. Zarember, K.A. and P.J. Godowski, *Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines.* J Immunol, 2002. **168**(2): p. 554-61.
26. Hornung, V., et al., *Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides.* J Immunol, 2002. **168**(9): p. 4531-7.
27. Wesch, D., et al., *Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes.* J Immunol, 2006. **176**(3): p. 1348-54.
28. Komai-Koma, M., et al., *TLR2 is expressed on activated T cells as a costimulatory receptor.* Proc Natl Acad Sci U S A, 2004. **101**(9): p. 3029-34.
29. Crellin, N.K., et al., *Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells.* J Immunol, 2005. **175**(12): p. 8051-9.
30. Jin, B., et al., *The effects of TLR activation on T-cell development and differentiation.* Clin Dev Immunol, 2012. **2012**: p. 836485.
31. Caramalho, I., et al., *Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide.* J Exp Med, 2003. **197**(4): p. 403-11.
32. Kulkarni, R., S. Behboudi, and S. Sharif, *Insights into the role of Toll-like receptors in modulation of T cell responses.* Cell Tissue Res, 2011. **343**(1): p. 141-52.
33. Salem, M.L., *Triggering of toll-like receptor signaling pathways in T cells contributes to the anti-tumor efficacy of T cell responses.* Immunol Lett, 2011. **137**(1-2): p. 9-14.
34. Liu, G., L. Zhang, and Y. Zhao, *Modulation of immune responses through direct activation of Toll-like receptors to T cells.* Clin Exp Immunol, 2010. **160**(2): p. 168-75.
35. Nyirenda, M.H., et al., *TLR2 stimulation drives human naive and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function.* J Immunol, 2011. **187**(5): p. 2278-90.
36. Reynolds, J.M., et al., *Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease.* Immunity, 2010. **32**(5): p. 692-702.
37. Chen, X., et al., *Engagement of Toll-like receptor 2 on CD4(+) T cells facilitates local immune responses in patients with tuberculous pleurisy.* J Infect Dis, 2009. **200**(3): p. 399-408.
38. Reynolds, J.M., et al., *Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation.* Proc Natl Acad Sci U S A, 2012. **109**(32): p. 13064-9.
39. Gonzalez-Navajas, J.M., et al., *TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice.* J Clin Invest, 2010. **120**(2): p. 570-81.
40. Zanin-Zhorov, A., et al., *Cutting edge: T cells respond to lipopolysaccharide innately via TLR4 signaling.* J Immunol, 2007. **179**(1): p. 41-4.
41. Funderburg, N., et al., *Toll-like receptor ligands induce human T cell activation and death, a model for HIV pathogenesis.* PLoS One, 2008. **3**(4): p. e1915.
42. Meyer, T., et al., *poly(I:C) costimulation induces a stronger antiviral chemokine and granzyme B release in human CD4 T cells than CD28 costimulation.* J Leukoc Biol, 2012. **92**(4): p. 765-74.
43. Gelman, A.E., et al., *Toll-like receptor ligands directly promote activated CD4+ T cell survival.* J Immunol, 2004. **172**(10): p. 6065-73.
44. Holm, C.K., et al., *TLR3 ligand polyinosinic:polycytidylic acid induces IL-17A and IL-21 synthesis in human Th cells.* J Immunol, 2009. **183**(7): p. 4422-31.
45. Cottalorda, A., et al., *TLR2 engagement on CD8 T cells lowers the threshold for optimal antigen-induced T cell activation.* Eur J Immunol, 2006. **36**(7): p. 1684-93.
46. Kappler, J., et al., *The mouse T cell receptor: comparison of MHC-restricted receptors on two T cell hybridomas.* Cell, 1983. **34**(3): p. 727-37.
47. Al-Lazikani, B., A.M. Lesk, and C. Chothia, *Canonical structures for the hypervariable regions of T cell alphabeta receptors.* J Mol Biol, 2000. **295**(4): p. 979-95.
48. Love, P.E. and S.M. Hayes, *ITAM-mediated signaling by the T-cell antigen receptor.* Cold Spring Harb Perspect Biol, 2010. **2**(6): p. a002485.

49. van der Merwe, P.A. and O. Dushek, *Mechanisms for T cell receptor triggering.* Nat Rev Immunol, 2011. **11**(1): p. 47-55.
50. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T cell activation.* Annu Rev Immunol, 2009. **27**: p. 591-619.
51. Capone, M., et al., *TCR beta and TCR alpha gene enhancers confer tissue- and stage-specificity on V(D)J recombination events.* EMBO J, 1993. **12**(11): p. 4335-46.
52. Davis, M.M., et al., *A murine T cell receptor gene complex: isolation, structure and rearrangement.* Immunol Rev, 1984. **81**: p. 235-58.
53. Thomas, D.W. and E.M. Shevach, *Nature of the antigenic complex recognized by T lymphocytes II. T-cell activation by direct modification of macrophage histocompatibility antigens.* J Exp Med, 1977. **145**(4): p. 907-15.
54. Zinkernagel, R.M. and P.C. Doherty, *H-2 compatability requirement for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. Different cytotoxic T-cell specificities are associated with structures coded for in H-2K or H-2D.* J Exp Med, 1975. **141**(6): p. 1427-36.
55. Bryant, P.W., et al., *Proteolysis and antigen presentation by MHC class II molecules.* Adv Immunol, 2002. **80**: p. 71-114.
56. Yewdell, J.W. and S.M. Haeryfar, *Understanding presentation of viral antigens to CD8+ T cells in vivo: the key to rational vaccine design.* Annu Rev Immunol, 2005. **23**: p. 651-82.
57. Trombetta, E.S. and I. Mellman, *Cell biology of antigen processing in vitro and in vivo.* Annu Rev Immunol, 2005. **23**: p. 975-1028.
58. Segura, E. and J.A. Villadangos, *Antigen presentation by dendritic cells in vivo.* Curr Opin Immunol, 2009. **21**(1): p. 105-10.
59. Heath, W.R. and F.R. Carbone, *Dendritic cell subsets in primary and secondary T cell responses at body surfaces.* Nat Immunol, 2009. **10**(12): p. 1237-44.
60. Guermonprez, P., et al., *Antigen presentation and T cell stimulation by dendritic cells.* Annu Rev Immunol, 2002. **20**: p. 621-67.
61. Thery, C. and S. Amigorena, *The cell biology of antigen presentation in dendritic cells.* Curr Opin Immunol, 2001. **13**(1): p. 45-51.
62. Germain, R.N., *T-cell development and the CD4-CD8 lineage decision.* Nat Rev Immunol, 2002. **2**(5): p. 309-22.
63. Teh, H.S., et al., *Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells.* Nature, 1988. **335**(6187): p. 229-33.
64. Page, D.M., et al., *Two signals are required for negative selection of CD4+CD8+ thymocytes.* J Immunol, 1993. **151**(4): p. 1868-80.
65. Klein, L., et al., *Antigen presentation in the thymus for positive selection and central tolerance induction.* Nat Rev Immunol, 2009. **9**(12): p. 833-44.
66. Gunzer, M., et al., *Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential.* Immunity, 2000. **13**(3): p. 323-32.
67. Wang, J.H. and M.J. Eck, *Assembling atomic resolution views of the immunological synapse.* Curr Opin Immunol, 2003. **15**(3): p. 286-93.
68. Dustin, M.L., *T-cell activation through immunological synapses and kinapses.* Immunol Rev, 2008. **221**: p. 77-89.
69. Quill, H. and R.H. Schwartz, *Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness.* J Immunol, 1987. **138**(11): p. 3704-12.
70. Bour-Jordan, H. and J.A. Blueston, *CD28 function: a balance of costimulatory and regulatory signals.* J Clin Immunol, 2002. **22**(1): p. 1-7.
71. Acuto, O. and F. Michel, *CD28-mediated co-stimulation: a quantitative support for TCR signalling.* Nat Rev Immunol, 2003. **3**(12): p. 939-51.
72. Zhou, X.Y., et al., *Molecular mechanisms underlying differential contribution of CD28 versus non-CD28 costimulatory molecules to IL-2 promoter activation.* J Immunol, 2002. **168**(8): p. 3847-54.
73. Thomas, R., *Signal 3 and its role in autoimmunity.* Arthritis Res Ther, 2004. **6**(1): p. 26-27.

74. Pepper, M. and M.K. Jenkins, *Origins of CD4(+) effector and central memory T cells.* Nat Immunol, 2011. **12**(6): p. 467-71.
75. O'Shea, J.J. and W.E. Paul, *Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells.* Science, 2010. **327**(5969): p. 1098-102.
76. Manetti, R., et al., *Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells.* J Exp Med, 1993. **177**(4): p. 1199-204.
77. Brombacher, F., R.A. Kastelein, and G. Alber, *Novel IL-12 family members shed light on the orchestration of Th1 responses.* Trends Immunol, 2003. **24**(4): p. 207-12.
78. Romagnani, S., *Lymphokine production by human T cells in disease states.* Annu Rev Immunol, 1994. **12**: p. 227-57.
79. Billiau, A. and P. Matthys, *Interferon-gamma: a historical perspective.* Cytokine Growth Factor Rev, 2009. **20**(2): p. 97-113.
80. Soyka, M.B., et al., *Defective epithelial barrier in chronic rhinosinusitis: the regulation of tight junctions by IFN-gamma and IL-4.* J Allergy Clin Immunol, 2012. **130**(5): p. 1087-1096 e10.
81. Rebane, A., et al., *Mechanisms of IFN-gamma-induced apoptosis of human skin keratinocytes in patients with atopic dermatitis.* J Allergy Clin Immunol, 2012. **129**(5): p. 1297-306.
82. Seder, R.A., et al., *The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice.* J Exp Med, 1992. **176**(4): p. 1091-8.
83. Farrar, J.D., H. Asnagli, and K.M. Murphy, *T helper subset development: roles of instruction, selection, and transcription.* J Clin Invest, 2002. **109**(4): p. 431-5.
84. Hasnain, S.Z., et al., *Muc5ac: a critical component mediating the rejection of enteric nematodes.* J Exp Med, 2011. **208**(5): p. 893-900.
85. Cliffe, L.J., et al., *Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion.* Science, 2005. **308**(5727): p. 1463-5.
86. Anthony, R.M., et al., *Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites.* Nat Med, 2006. **12**(8): p. 955-60.
87. Zhao, A., et al., *Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves.* J Immunol, 2003. **171**(2): p. 948-54.
88. Palm, N.W., R.K. Rosenstein, and R. Medzhitov, *Allergic host defences.* Nature, 2012. **484**(7395): p. 465-72.
89. Annunziato, F., et al., *Phenotypic and functional features of human Th17 cells.* J Exp Med, 2007. **204**(8): p. 1849-61.
90. Acosta-Rodriguez, E.V., et al., *Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells.* Nat Immunol, 2007. **8**(6): p. 639-46.
91. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nat Immunol, 2005. **6**(11): p. 1123-32.
92. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
93. Yang, X.O., et al., *STAT3 regulates cytokine-mediated generation of inflammatory helper T cells.* J Biol Chem, 2007. **282**(13): p. 9358-63.
94. Rutz, S., C. Eidenschenk, and W. Ouyang, *IL-22, not simply a Th17 cytokine.* Immunol Rev, 2013. **252**(1): p. 116-32.
95. Annunziato, F., et al., *Defining the human T helper 17 cell phenotype.* Trends Immunol, 2012. **33**(10): p. 505-12.
96. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
97. Dardalhon, V., et al., *IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells.* Nat Immunol, 2008. **9**(12): p. 1347-55.
98. Veldhoen, M., et al., *Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset.* Nat Immunol, 2008. **9**(12): p. 1341-6.

99. Schmitt, E., et al., *IL-9 production of naive CD4+ T cells depends on IL-2, is synergistically enhanced by a combination of TGF-beta and IL-4, and is inhibited by IFN-gamma.* J Immunol, 1994. **153**(9): p. 3989-96.
100. Chang, H.C., et al., *The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation.* Nat Immunol, 2010. **11**(6): p. 527-34.
101. Staudt, V., et al., *Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells.* Immunity, 2010. **33**(2): p. 192-202.
102. Licona-Limon, P., et al., *Th9 Cells Drive Host Immunity against Gastrointestinal Worm Infection.* Immunity, 2013. **39**(4): p. 744-57.
103. Purwar, R., et al., *Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells.* Nat Med, 2012. **18**(8): p. 1248-53.
104. Lu, Y., et al., *Th9 cells promote antitumor immune responses in vivo.* J Clin Invest, 2012. **122**(11): p. 4160-71.
105. Zhou, Y., et al., *IL-9 promotes Th17 cell migration into the central nervous system via CC chemokine ligand-20 produced by astrocytes.* J Immunol, 2011. **186**(7): p. 4415-21.
106. Jones, C.P., et al., *Activin A and TGF-beta promote T(H)9 cell-mediated pulmonary allergic pathology.* J Allergy Clin Immunol, 2012. **129**(4): p. 1000-10 e3.
107. Eyerich, S. and C.E. Zielinski, *Defining Th-cell subsets in a classical and tissue-specific manner: Examples from the skin.* Eur J Immunol, 2014. **44**(12): p. 3475-83.
108. Dumoutier, L., et al., *Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor.* Proc Natl Acad Sci U S A, 2000. **97**(18): p. 10144-9.
109. Dumoutier, L., et al., *IL-TIF/IL-22: genomic organization and mapping of the human and mouse genes.* Genes Immun, 2000. **1**(8): p. 488-94.
110. Dumoutier, L., J. Louahed, and J.C. Renauld, *Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9.* J Immunol, 2000. **164**(4): p. 1814-9.
111. Rutz, S., X. Wang, and W. Ouyang, *The IL-20 subfamily of cytokines--from host defence to tissue homeostasis.* Nat Rev Immunol, 2014. **14**(12): p. 783-95.
112. Xie, M.H., et al., *Interleukin (IL)-22, a novel human cytokine that signals through the interferon receptor-related proteins CRF2-4 and IL-22R.* J Biol Chem, 2000. **275**(40): p. 31335-9.
113. Nagem, R.A., et al., *Crystal structure of recombinant human interleukin-22.* Structure, 2002. **10**(8): p. 1051-62.
114. de Oliveira Neto, M., et al., *Interleukin-22 Forms Dimers that are Recognized by Two Interleukin-22R1 Receptor Chains().* Biophysical Journal, 2008. **94**(5): p. 1754-1765.
115. Xu, T., N.J. Logsdon, and M.R. Walter, *Structure of insect-cell-derived IL-22.* Acta Crystallogr D Biol Crystallogr, 2005. **61**(Pt 7): p. 942-50.
116. Li, J., et al., *Temporal associations between interleukin 22 and the extracellular domains of IL-22R and IL-10R2.* International Immunopharmacology, 2004. **4**(5): p. 693-708.
117. Kotenko, S.V., et al., *Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes.* J Biol Chem, 2001. **276**(4): p. 2725-32.
118. Logsdon, N.J., et al., *The IL-10R2 binding hot spot on IL-22 is located on the N-terminal helix and is dependent on N-linked glycosylation.* J Mol Biol, 2004. **342**(2): p. 503-14.
119. Jones, B.C., N.J. Logsdon, and M.R. Walter, *Structure of IL-22 bound to its high-affinity IL-22R1 chain.* Structure, 2008. **16**(9): p. 1333-44.
120. Logsdon, N.J., et al., *Comparison of interleukin-22 and interleukin-10 soluble receptor complexes.* J Interferon Cytokine Res, 2002. **22**(11): p. 1099-112.
121. Ouyang, W., et al., *Regulation and functions of the IL-10 family of cytokines in inflammation and disease.* Annu Rev Immunol, 2011. **29**: p. 71-109.
122. Wang, M., et al., *Interleukin 24 (MDA-7/MOB-5) signals through two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2.* J Biol Chem, 2002. **277**(9): p. 7341-7.

123. Dumoutier, L., et al., *Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types.* J Immunol, 2001. **167**(7): p. 3545-9.
124. Lejeune, D., et al., *Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP kinase pathways in a rat hepatoma cell line. Pathways that are shared with and distinct from IL-10.* J Biol Chem, 2002. **277**(37): p. 33676-82.
125. Mitra, A., S.K. Raychaudhuri, and S.P. Raychaudhuri, *IL-22 induced cell proliferation is regulated by PI3K/Akt/mTOR signaling cascade.* Cytokine, 2012. **60**(1): p. 38-42.
126. Wolk, K., et al., *Cutting edge: immune cells as sources and targets of the IL-10 family members?* J Immunol, 2002. **168**(11): p. 5397-402.
127. Wolk, K., et al., *IL-22 increases the innate immunity of tissues.* Immunity, 2004. **21**(2): p. 241-54.
128. Weiss, B., *Cloning of murine IL-22 receptor alpha 2 and comparison with its human counterpart.* Genes Immun., 2004. **5**: p. 330-336.
129. Kotenko, S.V., et al., *Identification, cloning, and characterization of a novel soluble receptor that binds IL-22 and neutralizes its activity.* J Immunol, 2001. **166**(12): p. 7096-103.
130. Gruenberg, B.H., et al., *A novel, soluble homologue of the human IL-10 receptor with preferential expression in placenta.* Genes Immun, 2001. **2**(6): p. 329-34.
131. Dumoutier, L., et al., *Cloning and characterization of IL-22 binding protein, a natural antagonist of IL-10-related T cell-derived inducible factor/IL-22.* J Immunol, 2001. **166**(12): p. 7090-5.
132. Martin, J.C., *Interleukin-22 binding protein (IL-22BP) is constitutively expressed by a subset of conventional dendritic cells and is strongly induced by retinoic acid.* Mucosal Immunol., 2013.
133. Huber, S., *IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine.* Nature, 2012. **491**: p. 259-263.
134. Wei, C.C., et al., *Cloning and characterization of mouse IL-22 binding protein.* Genes Immun., 2003. **4**: p. 204-211.
135. Xu, W., et al., *A soluble class II cytokine receptor, IL-22RA2, is a naturally occurring IL-22 antagonist.* Proc Natl Acad Sci U S A, 2001. **98**(17): p. 9511-6.
136. Wu, P.W., *IL-22R, IL-10R2, and IL-22BP binding sites are topologically juxtaposed on adjacent and overlapping surfaces of IL-22.* J. Mol. Biol., 2008. **382**: p. 1168-1183.
137. de Moura, P.R., *Crystal structure of a soluble decoy receptor IL-22BP bound to interleukin-22.* FEBS Lett., 2009. **583**: p. 1072-1077.
138. Duhen, T., et al., *Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells.* Nature Immunol., 2009. **10**: p. 857-863.
139. Volpe, E., et al., *Multiparametric analysis of cytokine-driven human Th17 differentiation reveals a differential regulation of IL-17 and IL-22 production.* Blood, 2009. **114**(17): p. 3610-4.
140. Gurney, A.L., *IL-22, a Th1 cytokine that targets the pancreas and select other peripheral tissues.* Int Immunopharmacol, 2004. **4**(5): p. 669-77.
141. Chung, Y., *Expression and regulation of IL-22 in the IL-17-producing CD4+ T lymphocytes.* Cell Res., 2006. **16**: p. 902-907.
142. Zheng, Y., *Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis.* Nature, 2007. **445**: p. 648-651.
143. Liang, S.C., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J. Exp. Med., 2006. **203**: p. 2271-2279.
144. Ghoreschi, K., et al., *Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling.* Nature, 2010. **467**(7318): p. 967-71.
145. Rutz, S., *Transcription factor c-Maf mediates the TGF-[beta]-dependent suppression of IL-22 production in TH17 cells.* Nature Immunol., 2011. **12**: p. 1238-1245.
146. Trifari, S., et al., *Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from TH-17, TH1 and TH2 cells.* Nature Immunol., 2009. **10**: p. 864-871.
147. Eyerich, S., et al., *Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling.* J Clin Invest, 2009. **119**(12): p. 3573-85.

148. Basu, R., *Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria.* Immunity, 2012. **37**: p. 1061-1075.
149. Fujita, H., et al., *Human Langerhans cells induce distinct IL-22-producing CD4+ T cells lacking IL-17 production.* Proc Natl Acad Sci U S A, 2009. **106**(51): p. 21795-800.
150. de Jong, A., et al., *CD1a-autoreactive T cells are a normal component of the human alphabeta T cell repertoire.* Nat Immunol, 2010. **11**(12): p. 1102-9.
151. Veldhoen, M., et al., *The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins.* Nature, 2008. **453**(7191): p. 106-9.
152. Liu, Y., et al., *Interleukin-21 induces the differentiation of human Tc22 cells via phosphorylation of signal transducers and activators of transcription.* Immunology, 2011. **132**(4): p. 540-8.
153. Ciric, B., et al., *IL-23 drives pathogenic IL-17-producing CD8+ T cells.* J Immunol, 2009. **182**(9): p. 5296-305.
154. Hamada, H., et al., *Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge.* J Immunol, 2009. **182**(6): p. 3469-81.
155. Kondo, T., et al., *Cutting edge: Phenotypic characterization and differentiation of human CD8+ T cells producing IL-17.* J Immunol, 2009. **182**(4): p. 1794-8.
156. Crellin, N.K., et al., *Regulation of cytokine secretion in human CD127(+) LTi-like innate lymphoid cells by Toll-like receptor 2.* Immunity, 2010. **33**(5): p. 752-64.
157. Mabuchi, T., T. Takekoshi, and S.T. Hwang, *Epidermal CCR6+ gammadelta T cells are major producers of IL-22 and IL-17 in a murine model of psoriasiform dermatitis.* J Immunol, 2011. **187**(10): p. 5026-31.
158. Martin, B., et al., *Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals.* Immunity, 2009. **31**(2): p. 321-30.
159. Paget, C., et al., *Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages.* J Biol Chem, 2012. **287**(12): p. 8816-29.
160. Goto, M., et al., *Murine NKT cells produce Th17 cytokine interleukin-22.* Cell Immunol, 2009. **254**(2): p. 81-4.
161. Doisne, J.M., et al., *Cutting edge: crucial role of IL-1 and IL-23 in the innate IL-17 response of peripheral lymph node NK1.1- invariant NKT cells to bacteria.* J Immunol, 2011. **186**(2): p. 662-6.
162. Zheng, Y., et al., *Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens.* Nat Med, 2008. **14**(3): p. 282-9.
163. Cella, M., et al., *A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity.* Nature, 2009. **457**(7230): p. 722-5.
164. Cupedo, T., et al., *Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells.* Nat Immunol, 2009. **10**(1): p. 66-74.
165. Takatori, H., et al., *Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22.* J Exp Med, 2009. **206**(1): p. 35-41.
166. Satoh-Takayama, N., et al., *Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense.* Immunity, 2008. **29**(6): p. 958-70.
167. Zindl, C.L., et al., *IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis.* Proc Natl Acad Sci U S A, 2013. **110**(31): p. 12768-73.
168. Mashiko, S., et al., *Human mast cells are major IL-22 producers in patients with psoriasis and atopic dermatitis.* J Allergy Clin Immunol, 2015.
169. Aujla, S.J., et al., *IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia.* Nat Med, 2008. **14**(3): p. 275-81.
170. Sekikawa, A., et al., *Involvement of the IL-22/REG Ialpha axis in ulcerative colitis.* Lab Invest, 2010. **90**(3): p. 496-505.
171. Wolk, K., et al., *IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis.* Eur J Immunol, 2006. **36**(5): p. 1309-23.

172. Boniface, K., et al., *IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes.* J Immunol, 2005. **174**(6): p. 3695-702.
173. Kumar, P., et al., *IL-22 from conventional NK cells is epithelial regenerative and inflammation protective during influenza infection.* Mucosal Immunol, 2013. **6**(1): p. 69-82.
174. Wolk, K., et al., *The Th17 cytokine IL-22 induces IL-20 production in keratinocytes: a novel immunological cascade with potential relevance in psoriasis.* Eur J Immunol, 2009. **39**(12): p. 3570-81.
175. Wolk, K., et al., *IL-22 and IL-20 are key mediators of the epidermal alterations in psoriasis while IL-17 and IFN-gamma are not.* J Mol Med (Berl), 2009. **87**(5): p. 523-36.
176. Boniface, K., et al., *A role for T cell-derived interleukin 22 in psoriatic skin inflammation.* Clin Exp Immunol, 2007. **150**(3): p. 407-15.
177. Nograles, K.E., et al., *IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells.* J Allergy Clin Immunol, 2009. **123**(6): p. 1244-52 e2.
178. Res, P.C., et al., *Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis.* PLoS One, 2010. **5**(11): p. e14108.
179. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
180. Tohyama, M., et al., *IFN-alpha enhances IL-22 receptor expression in keratinocytes: a possible role in the development of psoriasis.* J Invest Dermatol, 2012. **132**(7): p. 1933-5.
181. Tohyama, M., et al., *IL-17 and IL-22 mediate IL-20 subfamily cytokine production in cultured keratinocytes via increased IL-22 receptor expression.* Eur J Immunol, 2009. **39**(10): p. 2779-88.
182. Teraki, Y., A. Sakurai, and S. Izaki, *IL-13/IL-22-coproducing T cells, a novel subset, are increased in atopic dermatitis.* J Allergy Clin Immunol, 2013. **132**(4): p. 971-4.
183. Hayashida, S., et al., *Significant correlation of serum IL-22 levels with CCL17 levels in atopic dermatitis.* J Dermatol Sci, 2011. **61**(1): p. 78-9.
184. Andoh, A., et al., *Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts.* Gastroenterology, 2005. **129**(3): p. 969-84.
185. Wolk, K., et al., *IL-22 induces lipopolysaccharide-binding protein in hepatocytes: a potential systemic role of IL-22 in Crohn's disease.* J Immunol, 2007. **178**(9): p. 5973-81.
186. Zenewicz, L.A., et al., *IL-22 deficiency alters colonic microbiota to be transmissible and colitogenic.* J Immunol, 2013. **190**(10): p. 5306-12.
187. Sugimoto, K., et al., *IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis.* J Clin Invest, 2008. **118**(2): p. 534-44.
188. Pickert, G., et al., *STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing.* J Exp Med, 2009. **206**(7): p. 1465-72.
189. Kamanaka, M., et al., *Memory/effector (CD45RB(lo)) CD4 T cells are controlled directly by IL-10 and cause IL-22-dependent intestinal pathology.* J Exp Med, 2011. **208**(5): p. 1027-40.
190. Farfariello, V., et al., *IL-22 mRNA in peripheral blood mononuclear cells from allergic rhinitic and asthmatic pediatric patients.* Pediatr Allergy Immunol, 2011. **22**(4): p. 419-23.
191. Besnard, A.G., et al., *Dual Role of IL-22 in allergic airway inflammation and its cross-talk with IL-17A.* Am J Respir Crit Care Med, 2011. **183**(9): p. 1153-63.
192. Nakagome, K., et al., *High expression of IL-22 suppresses antigen-induced immune responses and eosinophilic airway inflammation via an IL-10-associated mechanism.* J Immunol, 2011. **187**(10): p. 5077-89.
193. Taube, C., et al., *IL-22 is produced by innate lymphoid cells and limits inflammation in allergic airway disease.* PLoS One, 2011. **6**(7): p. e21799.
194. Takahashi, K., et al., *IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation.* J Allergy Clin Immunol, 2011. **128**(5): p. 1067-76 e1-6.

195. Sonnenberg, G.F., L.A. Fouser, and D. Artis, *Functional biology of the IL-22-IL-22R pathway in regulating immunity and inflammation at barrier surfaces.* Adv Immunol, 2010. **107**: p. 1-29.
196. Ikeuchi, H., et al., *Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine.* Arthritis Rheum, 2005. **52**(4): p. 1037-46.
197. da Rocha, L.F., Jr., et al., *Increased serum interleukin 22 in patients with rheumatoid arthritis and correlation with disease activity.* J Rheumatol, 2012. **39**(7): p. 1320-5.
198. Zhang, L., et al., *Elevated Th22 cells correlated with Th17 cells in patients with rheumatoid arthritis.* J Clin Immunol, 2011. **31**(4): p. 606-14.
199. Leipe, J., et al., *Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis.* Ann Rheum Dis, 2011. **70**(8): p. 1453-7.
200. Kim, K.W., et al., *Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts.* Arthritis Rheum, 2012. **64**(4): p. 1015-23.
201. Pan, H., et al., *Hydrodynamic gene delivery of interleukin-22 protects the mouse liver from concanavalin A-, carbon tetrachloride-, and Fas ligand-induced injury via activation of STAT3.* Cell Mol Immunol, 2004. **1**(1): p. 43-9.
202. Schulz, S.M., et al., *Protective immunity to systemic infection with attenuated Salmonella enterica serovar enteritidis in the absence of IL-12 is associated with IL-23-dependent IL-22, but not IL-17.* J Immunol, 2008. **181**(11): p. 7891-901.
203. Mastelic, B., et al., *IL-22 Protects Against Liver Pathology and Lethality of an Experimental Blood-Stage Malaria Infection.* Front Immunol, 2012. **3**: p. 85.
204. Chestovich, P.J., et al., *Interleukin-22: implications for liver ischemia-reperfusion injury.* Transplantation, 2012. **93**(5): p. 485-92.
205. Ki, S.H., et al., *Interleukin-22 treatment ameliorates alcoholic liver injury in a murine model of chronic-binge ethanol feeding: role of signal transducer and activator of transcription 3.* Hepatology, 2010. **52**(4): p. 1291-300.
206. Liang, S.C., et al., *IL-22 induces an acute-phase response.* J Immunol, 2010. **185**(9): p. 5531-8.
207. Feng, D., et al., *Interleukin-22 promotes proliferation of liver stem/progenitor cells in mice and patients with chronic hepatitis B virus infection.* Gastroenterology, 2012. **143**(1): p. 188-98 e7.
208. Aggarwal, S., et al., *Acinar cells of the pancreas are a target of interleukin-22.* J Interferon Cytokine Res, 2001. **21**(12): p. 1047-53.
209. Shioya, M., et al., *Interleukin 22 receptor 1 expression in pancreas islets.* Pancreas, 2008. **36**(2): p. 197-9.
210. Hill, T., et al., *The involvement of interleukin-22 in the expression of pancreatic beta cell regenerative Reg genes.* Cell Regen (Lond), 2013. **2**(1): p. 2.
211. Feng, D., et al., *Interleukin-22 ameliorates cerulein-induced pancreatitis in mice by inhibiting the autophagic pathway.* Int J Biol Sci, 2012. **8**(2): p. 249-57.
212. Xue, J., D.T. Nguyen, and A. Habtezion, *Aryl hydrocarbon receptor regulates pancreatic IL-22 production and protects mice from acute pancreatitis.* Gastroenterology, 2012. **143**(6): p. 1670-80.
213. Graham, A.C., et al., *IL-22 production is regulated by IL-23 during Listeria monocytogenes infection but is not required for bacterial clearance or tissue protection.* PLoS One, 2011. **6**(2): p. e17171.
214. Wilson, M.S., *Redundant and pathogenic roles for IL-22 in mycobacterial, protozoan, and helminth infections.* J. Immunol., 2010. **184**: p. 4378-4390.
215. Lin, Y., *Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen Francisella tularensis.* Immunity, 2009. **31**: p. 799-810.
216. Kudva, A., et al., *Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice.* J Immunol, 2011. **186**(3): p. 1666-74.
217. Simonian, P.L., et al., *gammadelta T cells protect against lung fibrosis via IL-22.* J Exp Med, 2010. **207**(10): p. 2239-53.
218. Gessner, M.A., et al., *Dectin-1-dependent interleukin-22 contributes to early innate lung defense against Aspergillus fumigatus.* Infect Immun, 2012. **80**(1): p. 410-7.

219. De Luca, A., et al., *IL-22 defines a novel immune pathway of antifungal resistance.* Mucosal Immunol, 2010. **3**(4): p. 361-73.
220. Conti, H.R., et al., *Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis.* J Exp Med, 2009. **206**(2): p. 299-311.
221. Kagami, S., et al., *IL-23 and IL-17A, but not IL-12 and IL-22, are required for optimal skin host defense against Candida albicans.* J Immunol, 2010. **185**(9): p. 5453-62.
222. Puel, A., et al., *Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I.* J Exp Med, 2010. **207**(2): p. 291-7.
223. Pociask, D.A., et al., *IL-22 is essential for lung epithelial repair following influenza infection.* Am J Pathol, 2013. **182**(4): p. 1286-96.
224. Kim, C.J., et al., *A role for mucosal IL-22 production and Th22 cells in HIV-associated mucosal immunopathogenesis.* Mucosal Immunol, 2012. **5**(6): p. 670-80.
225. Ogra, P.L., *Mucosal immune response in the ear, nose and throat.* Pediatr Infect Dis J, 2000. **19**(5 Suppl): p. S4-8.
226. Perry, M. and A. Whyte, *Immunology of the tonsils.* Immunol Today, 1998. **19**(9): p. 414-21.
227. Hall, J., *Immunology of the lung and upper respiratory tract by John Bienenstock, McGraw-Hill Book Company, 1984. pound32.95 (xiv + 414 pages) ISBN 0 07 005215 8.* Immunol Today, 1984. **5**(10): p. 305.
228. Palomares, O., et al., *Induction and maintenance of allergen-specific FOXP3+ Treg cells in human tonsils as potential first-line organs of oral tolerance.* J Allergy Clin Immunol, 2012. **129**(2): p. 510-20, 520 e1-9.
229. Howie, A.J., *Scanning and transmission electron microscopy on the epithelium of human palatine tonsils.* J Pathol, 1980. **130**(2): p. 91-8.
230. Tang, X., et al., *Reticular crypt epithelium and intra-epithelial lymphoid cells in the hyperplastic human palatine tonsil: an immunohistochemical analysis.* Pathol Int, 1995. **45**(1): p. 34-44.
231. Jepson, M.A., et al., *Targeting to intestinal M cells.* J Anat, 1996. **189 (Pt 3)**: p. 507-16.
232. Nave, H., A. Gebert, and R. Pabst, *Morphology and immunology of the human palatine tonsil.* Anat Embryol (Berl), 2001. **204**(5): p. 367-73.
233. McClory, S., et al., *Evidence for a stepwise program of extrathymic T cell development within the human tonsil.* J Clin Invest, 2012. **122**(4): p. 1403-15.
234. Dunham, I., *An integrated encyclopedia of DNA elements in the human genome.* Nature, 2012. **489**(7414): p. 57-74.
235. Berretta, J. and A. Morillon, *Pervasive transcription constitutes a new level of eukaryotic genome regulation.* EMBO Rep, 2009. **10**(9): p. 973-82.
236. Jacquier, A., *The complex eukaryotic transcriptome: unexpected pervasive transcription and novel small RNAs.* Nat Rev Genet, 2009. **10**(12): p. 833-44.
237. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-54.
238. Lagos-Quintana, M., et al., *Identification of novel genes coding for small expressed RNAs.* Science, 2001. **294**(5543): p. 853-8.
239. Lee, Y., et al., *MicroRNA genes are transcribed by RNA polymerase II.* EMBO J, 2004. **23**(20): p. 4051-60.
240. Cai, X., C.H. Hagedorn, and B.R. Cullen, *Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs.* RNA, 2004. **10**(12): p. 1957-66.
241. Han, J., et al., *The Drosha-DGCR8 complex in primary microRNA processing.* Genes Dev, 2004. **18**(24): p. 3016-27.
242. Lee, Y., et al., *The nuclear RNase III Drosha initiates microRNA processing.* Nature, 2003. **425**(6956): p. 415-9.
243. Yi, R., et al., *Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs.* Genes Dev, 2003. **17**(24): p. 3011-6.
244. Hutvagner, G., et al., *A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA.* Science, 2001. **293**(5531): p. 834-8.

245. Yang, J.S., et al., *Widespread regulatory activity of vertebrate microRNA* species.* RNA, 2011. **17**(2): p. 312-26.
246. Grimson, A., et al., *MicroRNA targeting specificity in mammals: determinants beyond seed pairing.* Mol Cell, 2007. **27**(1): p. 91-105.
247. Pfaff, J. and G. Meister, *Argonaute and GW182 proteins: an effective alliance in gene silencing.* Biochem Soc Trans, 2013. **41**(4): p. 855-60.
248. Miranda, K.C., et al., *A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes.* Cell, 2006. **126**(6): p. 1203-17.
249. Monticelli, S., et al., *MicroRNA profiling of the murine hematopoietic system.* Genome Biol, 2005. **6**(8): p. R71.
250. Rossi, R.L., et al., *Distinct microRNA signatures in human lymphocyte subsets and enforcement of the naive state in CD4+ T cells by the microRNA miR-125b.* Nat Immunol, 2011. **12**(8): p. 796-803.
251. Chong, M.M., et al., *The RNAseIII enzyme Drosha is critical in T cells for preventing lethal inflammatory disease.* J Exp Med, 2008. **205**(9): p. 2005-17.
252. Muljo, S.A., et al., *Aberrant T cell differentiation in the absence of Dicer.* J Exp Med, 2005. **202**(2): p. 261-9.
253. Bronevetsky, Y., et al., *T cell activation induces proteasomal degradation of Argonaute and rapid remodeling of the microRNA repertoire.* J Exp Med, 2013. **210**(2): p. 417-32.
254. Sandberg, R., et al., *Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites.* Science, 2008. **320**(5883): p. 1643-7.
255. Jeker, L.T. and J.A. Bluestone, *MicroRNA regulation of T-cell differentiation and function.* Immunol Rev, 2013. **253**(1): p. 65-81.
256. Jiang, S., et al., *Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation.* Blood, 2011. **118**(20): p. 5487-97.
257. Xiao, C., et al., *Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes.* Nat Immunol, 2008. **9**(4): p. 405-14.
258. Li, Q.J., et al., *miR-181a is an intrinsic modulator of T cell sensitivity and selection.* Cell, 2007. **129**(1): p. 147-61.
259. Stittrich, A.B., et al., *The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes.* Nat Immunol, 2010. **11**(11): p. 1057-62.
260. Curtale, G., et al., *An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes.* Blood, 2010. **115**(2): p. 265-73.
261. Xue, Q., et al., *Human activated CD4(+) T lymphocytes increase IL-2 expression by downregulating microRNA-181c.* Mol Immunol, 2011. **48**(4): p. 592-9.
262. Kaminski, B.A., et al., *Reduced expression of NFAT-associated genes in UCB versus adult CD4+ T lymphocytes during primary stimulation.* Blood, 2003. **102**(13): p. 4608-17.
263. Fan, W., et al., *Identification of microRNA-31 as a novel regulator contributing to impaired interleukin-2 production in T cells from patients with systemic lupus erythematosus.* Arthritis Rheum, 2012. **64**(11): p. 3715-25.
264. Baumjohann, D. and K.M. Ansel, *MicroRNA-mediated regulation of T helper cell differentiation and plasticity.* Nat Rev Immunol, 2013. **13**(9): p. 666-78.
265. Steiner, D.F., et al., *MicroRNA-29 regulates T-box transcription factors and interferon-gamma production in helper T cells.* Immunity, 2011. **35**(2): p. 169-81.
266. Ma, F., et al., *The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma.* Nat Immunol, 2011. **12**(9): p. 861-9.
267. Smith, K.M., et al., *miR-29ab1 deficiency identifies a negative feedback loop controlling Th1 bias that is dysregulated in multiple sclerosis.* J Immunol, 2012. **189**(4): p. 1567-76.
268. Yang, L., et al., *miR-146a controls the resolution of T cell responses in mice.* J Exp Med, 2012. **209**(9): p. 1655-70.
269. Lu, L.F., et al., *Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses.* Cell, 2010. **142**(6): p. 914-29.

270. Huffaker, T.B., et al., *Epistasis between microRNAs 155 and 146a during T cell-mediated antitumor immunity.* Cell Rep, 2012. **2**(6): p. 1697-709.
271. Lu, L.F., et al., *Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein.* Immunity, 2009. **30**(1): p. 80-91.
272. Thai, T.H., et al., *Regulation of the germinal center response by microRNA-155.* Science, 2007. **316**(5824): p. 604-8.
273. Rodriguez, A., et al., *Requirement of bic/microRNA-155 for normal immune function.* Science, 2007. **316**(5824): p. 608-11.
274. Lu, T.X., et al., *MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-gamma pathway, Th1 polarization, and the severity of delayed-type hypersensitivity.* J Immunol, 2011. **187**(6): p. 3362-73.
275. Mattes, J., et al., *Antagonism of microRNA-126 suppresses the effector function of TH2 cells and the development of allergic airways disease.* Proc Natl Acad Sci U S A, 2009. **106**(44): p. 18704-9.
276. Guerau-de-Arellano, M., et al., *Micro-RNA dysregulation in multiple sclerosis favours pro-inflammatory T-cell-mediated autoimmunity.* Brain, 2011. **134**(Pt 12): p. 3578-89.
277. Sawant, D.V., et al., *The Bcl6 target gene microRNA-21 promotes Th2 differentiation by a T cell intrinsic pathway.* Mol Immunol, 2013. **54**(3-4): p. 435-42.
278. Du, C., et al., *MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis.* Nat Immunol, 2009. **10**(12): p. 1252-9.
279. Murugaiyan, G., et al., *Silencing microRNA-155 ameliorates experimental autoimmune encephalomyelitis.* J Immunol, 2011. **187**(5): p. 2213-21.
280. O'Connell, R.M., et al., *MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development.* Immunity, 2010. **33**(4): p. 607-19.
281. Oertli, M., et al., *MicroRNA-155 is essential for the T cell-mediated control of Helicobacter pylori infection and for the induction of chronic Gastritis and Colitis.* J Immunol, 2011. **187**(7): p. 3578-86.
282. Bluml, S., et al., *Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice.* Arthritis Rheum, 2011. **63**(5): p. 1281-8.
283. Mycko, M.P., et al., *MicroRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination.* Proc Natl Acad Sci U S A, 2012. **109**(20): p. E1248-57.
284. Akdis, M., et al., *TH17 and TH22 cells: a confusion of antimicrobial response with tissue inflammation versus protection.* J Allergy Clin Immunol, 2012. **129**(6): p. 1438-49; quiz1450-1.
285. Eyerich, S., et al., *IL-17 and IL-22: siblings, not twins.* Trends Immunol, 2010. **31**(9): p. 354-61.
286. Rutz, S., et al., *Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells.* Nat Immunol, 2011. **12**(12): p. 1238-45.
287. Duhen, T., et al., *Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells.* Nat Immunol, 2009. **10**(8): p. 857-63.
288. Trifari, S., et al., *Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells.* Nat Immunol, 2009. **10**(8): p. 864-71.
289. Takahashi, K., *IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation.* J. Allergy Clin. Immunol., 2011. **128**: p. 1067-1076.
290. Fogli, L.K., et al., *T cell-derived IL-17 mediates epithelial changes in the airway and drives pulmonary neutrophilia.* J Immunol, 2013. **191**(6): p. 3100-11.
291. Mizutani, N., T. Nabe, and S. Yoshino, *IL-17A promotes the exacerbation of IL-33-induced airway hyperresponsiveness by enhancing neutrophilic inflammation via CXCR2 signaling in mice.* J Immunol, 2014. **192**(4): p. 1372-84.
292. Makeyev, E.V. and T. Maniatis, *Multilevel regulation of gene expression by microRNAs.* Science, 2008. **319**(5871): p. 1789-90.

293. Takahashi, H., et al., *TGF-beta and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and constrains the plasticity of helper T cells.* Nat Immunol, 2012. **13**(6): p. 587-95.
294. Rebane, A. and C.A. Akdis, *MicroRNAs: Essential players in the regulation of inflammation.* Journal of Allergy and Clinical Immunology, 2013. **132**(1): p. 15-26.
295. Lu, T.X. and M.E. Rothenberg, *Diagnostic, functional, and therapeutic roles of microRNA in allergic diseases.* J Allergy Clin Immunol, 2013. **132**(1): p. 3-13; quiz 14.
296. Malmhall, C., et al., *MicroRNA-155 is essential for T(H)2-mediated allergen-induced eosinophilic inflammation in the lung.* J Allergy Clin Immunol, 2014. **133**(5): p. 1429-38, 1438 e1-7.
297. Akdis, C.A., et al., *A molecular basis for T cell suppression by IL-10: CD28-associated IL-10 receptor inhibits CD28 tyrosine phosphorylation and phosphatidylinositol 3-kinase binding.* FASEB J, 2000. **14**(12): p. 1666-8.
298. Tserel, L., et al., *MicroRNA Expression Profiles of Human Blood Monocyte-derived Dendritic Cells and Macrophages Reveal miR-511 as Putative Positive Regulator of Toll-like Receptor 4.* J Biol Chem, 2011. **286**(30): p. 26487-95.
299. Friedman, R.C., et al., *Most mammalian mRNAs are conserved targets of microRNAs.* Genome Res, 2009. **19**(1): p. 92-105.
300. Reimand, J., et al., *g:Profiler--a web-based toolset for functional profiling of gene lists from large-scale experiments.* Nucleic Acids Res, 2007. **35**(Web Server issue): p. W193-200.
301. Rebane, A., et al., *MicroRNA-146a alleviates chronic skin inflammation in atopic dermatitis through suppression of innate immune responses in keratinocytes.* J Allergy Clin Immunol, 2014. **134**(4): p. 836-847 e11.
302. Jeon, S.H., et al., *Characterization of the direct physical interaction of nc886, a cellular non-coding RNA, and PKR.* FEBS Lett, 2012. **586**(19): p. 3477-84.
303. Taganov, K.D., et al., *NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12481-6.
304. Okoye, I.S., et al., *Transcriptomics identified a critical role for Th2 cell-intrinsic miR-155 in mediating allergy and antihelminth immunity.* Proc Natl Acad Sci U S A, 2014. **111**(30): p. E3081-90.
305. Rubtsov, Y.P. and A.Y. Rudensky, *TGFbeta signalling in control of T-cell-mediated self-reactivity.* Nat Rev Immunol, 2007. **7**(6): p. 443-53.
306. Polyak, K., et al., *Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals.* Cell, 1994. **78**(1): p. 59-66.
307. Seitz, H., et al., *A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain.* Genome Res, 2004. **14**(9): p. 1741-8.
308. Manodoro, F., et al., *Loss of imprinting at the 14q32 domain is associated with microRNA overexpression in acute promyelocytic leukemia.* Blood, 2014. **123**(13): p. 2066-74.
309. Nadal, E., et al., *A MicroRNA cluster at 14q32 drives aggressive lung adenocarcinoma.* Clin Cancer Res, 2014. **20**(12): p. 3107-17.
310. Xu, T., et al., *MicroRNA-323-3p: a new biomarker and potential therapeutic target for rheumatoid arthritis.* Rheumatol Int, 2014. **34**(5): p. 721-2.
311. Pandis, I., et al., *Identification of microRNA-221/222 and microRNA-323-3p association with rheumatoid arthritis via predictions using the human tumour necrosis factor transgenic mouse model.* Ann Rheum Dis, 2012. **71**(10): p. 1716-23.
312. Farfariello, V., *IL-22 mRNA in peripheral blood mononuclear cells from allergic rhinitic and asthmatic pediatric patients.* Pediatr. Allergy Immunol., 2011. **22**: p. 419-423.
313. Chien, J.W., et al., *Increased IL-17A secreting CD4+ T cells, serum IL-17 levels and exhaled nitric oxide are correlated with childhood asthma severity.* Clin Exp Allergy, 2013. **43**(9): p. 1018-26.
314. Nurgazieva, D., et al., *TGF-beta1, but not bone morphogenetic proteins, activates Smad1/5 pathway in primary human macrophages and induces expression of proatherogenic genes.* J Immunol, 2015. **194**(2): p. 709-18.

315. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. **7**: p. 145-73.
316. Dudakov, J.A., A.M. Hanash, and M.R. van den Brink, *Interleukin-22: immunobiology and pathology.* Annu Rev Immunol, 2015. **33**: p. 747-85.
317. Wolk, K., *IL-22 and IL-20 are key mediators of the epidermal alterations in psoriasis while IL-17 and IFN-[gamma] are not.* J. Mol. Med., 2009. **87**: p. 523-536.
318. Wolk, K., *IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis.* Eur. J. Immunol., 2006. **36**: p. 1309-1323.
319. Schmitt, E., M. Klein, and T. Bopp, *Th9 cells, new players in adaptive immunity.* Trends Immunol, 2014. **35**(2): p. 61-8.
320. Corvaisier, M., et al., *IL-26 is overexpressed in rheumatoid arthritis and induces proinflammatory cytokine production and Th17 cell generation.* PLoS Biol, 2012. **10**(9): p. e1001395.
321. Meller, S., et al., *TH17 cells promote microbial killing and innate immune sensing of DNA via interleukin 26.* Nat Immunol, 2015. **16**(9): p. 970-9.
322. Yeste, A., et al., *IL-21 induces IL-22 production in CD4+ T cells.* Nat Commun, 2014. **5**: p. 3753.
323. Griffith, J.W., C.L. Sokol, and A.D. Luster, *Chemokines and chemokine receptors: positioning cells for host defense and immunity.* Annu Rev Immunol, 2014. **32**: p. 659-702.
324. Sonnenberg, G.F., et al., *Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A.* J Exp Med, 2010. **207**(6): p. 1293-305.
325. Moreira, A.P., et al., *CCR5-dependent regulatory T cell migration mediates fungal survival and severe immunosuppression.* J Immunol, 2008. **180**(5): p. 3049-56.
326. Yurchenko, E., et al., *CCR5-dependent homing of naturally occurring CD4+ regulatory T cells to sites of Leishmania major infection favors pathogen persistence.* J Exp Med, 2006. **203**(11): p. 2451-60.
327. Wysocki, C.A., et al., *Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease.* Blood, 2005. **106**(9): p. 3300-7.
328. Zhang, N., et al., *Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response.* Immunity, 2009. **30**(3): p. 458-69.
329. Germanov, E., et al., *Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells.* J Immunol, 2008. **181**(1): p. 81-91.
330. Scimone, M.L., et al., *CXCL12 mediates CCR7-independent homing of central memory cells, but not naive T cells, in peripheral lymph nodes.* J Exp Med, 2004. **199**(8): p. 1113-20.
331. Murtagh, F. and P. Legendre, *Ward's Hierarchical Agglomerative Clustering Method: Which Algorithms Implement Ward's Criterion?* Journal of Classification, 2014. **31**(3): p. 274-295.
332. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing.* Journal of the Royal Statistical Society. Series B (Methodological), 1995. **57**(1): p. 289-300.
333. Ferreira, L.S., et al., *Optimal clearance of Sporothrix schenckii requires an intact Th17 response in a mouse model of systemic infection.* Immunobiology, 2015. **220**(8): p. 985-92.
334. Wolk, K., *IL-22 increases the innate immunity of tissues.* Immunity, 2004. **21**: p. 241-254.
335. Zheng, Y., *Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens.* Nature Med., 2008. **14**: p. 282-289.
336. Sekikawa, A., *Involvement of the IL-22/REG I[alpha] axis in ulcerative colitis.* Lab Invest., 2010. **90**: p. 496-505.
337. Aujla, S.J., *IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia.* Nature Med., 2008. **14**: p. 275-281.
338. Boniface, K., *IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes.* J. Immunol., 2005. **174**: p. 3695-3702.
339. Wolk, K., *The Th17 cytokine IL-22 induces IL-20 production in keratinocytes: a novel immunological cascade with potential relevance in psoriasis.* Eur. J. Immunol., 2009. **39**: p. 3570-3581.

340. Teraki, Y., A. Sakurai, and S. Izaki, *IL-13/IL-22-coproducing T cells, a novel subset, are increased in atopic dermatitis.* J. Allergy Clin. Immunol., 2013. **132**: p. 971-974.
341. Geboes, L., *Proinflammatory role of the Th17 cytokine interleukin-22 in collagen-induced arthritis in C57BL/6 mice.* Arthritis Rheum., 2009. **60**: p. 390-395.
342. Munoz, M., *Interleukin (IL)-23 mediates Toxoplasma gondii-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17.* J. Exp. Med., 2009. **206**: p. 3047-3059.
343. Aslan, F.M., et al., *Engineered single-chain dimeric streptavidins with an unexpected strong preference for biotin-4-fluorescein.* Proc Natl Acad Sci U S A, 2005. **102**(24): p. 8507-12.
344. Manz, R., et al., *Analysis and sorting of live cells according to secreted molecules, relocated to a cell-surface affinity matrix.* Proc Natl Acad Sci U S A, 1995. **92**(6): p. 1921-5.
345. Sonnenberg, G.F., et al., *Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A.* The Journal of Experimental Medicine, 2010. **207**(6): p. 1293-1305.
346. Eyerich, K. and S. Eyerich, *Th22 cells in allergic disease.* Allergo J Int, 2015. **24**(1): p. 1-7.
347. Backert, I., et al., *STAT3 activation in Th17 and Th22 cells controls IL-22-mediated epithelial host defense during infectious colitis.* J Immunol, 2014. **193**(7): p. 3779-91.
348. Schluns, K.S. and L. Lefrancois, *Cytokine control of memory T-cell development and survival.* Nat Rev Immunol, 2003. **3**(4): p. 269-79.
349. Zenewicz, L.A. and R.A. Flavell, *Recent advances in IL-22 biology.* Int Immunol, 2011. **23**(3): p. 159-63.